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Phospholipase C-γ1 is a GEF for Rac1 to regulate EGF-induced cell motility

by

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in partial fulfillment of the requirements for the degree of

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Abstract

It is well established that EGF induces cell movement by activating EGFR kinase and EGFR autophosphorylation. At least two signaling pathways downstream of EGFR can be linked to cell movement. Phospholipase C-γ1 (PLC-γ1) has been implicated in the pathway responsible for the reorganization of the cytoskeleton. Alternatively, EGFR activation leads to membrane ruffling and reorganization of focal adhesions through activation of member of the Rho subfamily of GTP-binding proteins. Given that both PLC-γ1 and Rho GTPases regulates cell motility induced by EGF, it would be interesting to examine whether there is a direct functional linkage between PLC-γ1 and Rho GTPase in EGF-induced cell movement. Recently, it was shown that PLC-γ1 SH3 domain is a GEF for GTPases including PIKE and dynamin. Thus, it is possible that PLC-γ1 may regulate cell motility and cancer metastasis by regulating Rho GTPase activity through its SH3 domain GEF activity. We tested this possibility in this research. We showed that in response to EGF PLC-γ1 and Rac1 co-localize to the plasma membrane and interact with each other. The interaction between PLC-γ1 and Rac1 is mediated by PLC-γ1 SH3 domain and Rac1 proline rich motif ^{106}PNTP^{109}. We further showed that EGF-induced PLC-γ1 and Rac1 interaction resulted in the activation of Rac1, which suggest that PLC-γ1 is a GEF for Rac1 *in vivo*. Moreover, we demonstrated by *in vitro* GEF assay that PLC-γ1 SH3 domain is a strong and specific GEF for Rac1. Finally, we showed that the interaction between PLC-γ1 and Rac1 enhanced EGF-induced cell motility. We conclude that PLC-γ1 functions as a Rac1 GEF to mediate EGF-induced cell movement.

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Abbreviations

Arp	Actin-related protein
Cdc42	cell division cycle 42 homolog
DAG	diacylglycerol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ErbB	erythroblastic leukemia viral oncogene homolog
FBS	fetal bovine serum
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GEF	guanine nucleotide exchange factor
GF	growth factor
GFP	green fluorescent protein
Grab2	growth factor receptor bound protein 2
GTPase	guanosine triphosphatase
GTP	guanine triphosphate
HGF	hepatocyte Growth Factor
IF	immunofluorescence
IP	immunoprecipitation
IP_3	inositol 1, 4, 5-triphosphate
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kDa	Kilo Daltons
NGF	nerve growth factor
PAK	p21 activated kinase
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI3K	phosphatidyl-inositol-3-kinase
PIKE	PI3K enhancer
PLC-γ1	phospholipase C-γ1
PLD_2	phospholipase D2
PKB	protein kinase B
PKC	protein kinase C
PtdIns 4,5-P_2	phosphatidylinositol-4,5-bis-phosphate
PX	Phox homology
RasGAP	ras-GTPase activating protein
Rac1	ras-related C3 botulinum toxin substrate 1
Rac2	ras-related C3 botulinum toxin substrate 2
Rac3	ras-related C3 botulinum toxin substrate 3
RBD	Rho binding domain
RhoA	ras homolog gene family, member A
RTK	receptor tyrosine kinase

SH2	Src homology 2
SH3	Src homology 3
SHC	Src homologous and collagen like protein
TGF-α	transforming growth factors-alpha
WT	wild type
YFP	yellow fluorescent protein

Chapter 1

Introduction

1.1 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are involved in cell signaling pathways and play critical roles in normal physiological processes, such as embryogenesis, cell proliferation, cell differentiation, cell metabolism, cell anti-apoptosis and neurite outgrowth as well as cell motility (reviewed in Bergeron et al., 1995; Barbieri et al., 2004). RTKs are composed of an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. Characteristically, the extracellular domains are comprised of one or more identifiable structural motifs, including cysteine-rich regions, fibronectin III-like domains, immunoglobulin-like domains, cadherin-like domains, EGF-like domains, Factor VIII-like domains, kringle-like domains, glycine-rich regions, leucine-rich regions, acidic regions and discoidin-like domains. The transmembrane domain anchors the receptor in the plasma membrane. The cytoplasmic portion of RTKs consists of a juxtamembrane region (just after the transmembrane helix), followed by the tyrosine kinase catalytic domain and a carboxy-teminal regulatory region (Bergeron et al., 1995; Barbieri et al., 2004; Pawson et al., 2002; Schlessinger, 2000) (Figure.1.1). Activation of the kinase domain is achieved by ligand binding to the extracellular domain, which induces dimerization of the receptors. Receptors thus activated are able to autophosphorylate tyrosine residues outside the catalytic domain via cross-phosphorylation. The auto-phosphorylation stabilizes the active receptor conformation and also creates phosphotyrosine docking sites for proteins which transduct intracellular cues. Signaling proteins which bind to the intracellular domain of RTKs in a phosphotyrosine-dependent manner include

RasGAP, PI3-kinase, PLC-γ1, phosphotyrosine phosphatase SHP and adaptor proteins such as Shc, Grb2 and Crk (Kovalenko et al., 1994; Levitzki, 1999).

1.2 Epidermal Growth Factor Receptors

Epidermal growth factor (EGF) receptor (EGFR, ErbB1 or HER1) is the first identified RTK (Carpenter et al., 1978; Carpenter et al., 1979; Hunter and Cooper, 1981; Cohen et al., 1980) and therefore one of the most extensively characterized. Since the discovery of EGFR, three additional members of ErbB family had been identified, designated ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4) (Ullrich et al., 1984; Coussens et al., 1985; Kraus et al., 1989; Plowman et al., 1993). Activation of the EGFR signals many biological responses including cell proliferation, cell differentiation, cell survival and cell motility (Pawson, 1995; Wells et al., 1998; Ware et al., 1998). Furthermore, overexpression or dysregulation of the EGFR family members has been implicated in a variety of human cancers, such as mammary carcinoma, squamous carcinoma, and glioblastomas (Yarden and Sliwkowski, 2001; Yarden et al., 2001b; Yarden, 2001; Yarden et al., 2001; Frykberg et al., 1983).

EGFR is a 170 kDa transmembrane glycoprotein of single polypeptide chain. Similar to other RTKS, EGFRs possess an extracellular ligand binding domain, a transmembrane domain, a juxtamembrane region, a tyrosine kinase domain and a carboxy-terminal regulatory domain (Figure 1.2). The heavily glycosylated 622-amino extracellular domain contains two cysteine rich regions (domains I and III) and is responsible for ligand binding. It can bind at least 6 ligands, EGF, Heparin-binding EGF (HB-EGF), Transforming Growth Factors-α (TGF-α), amphiregulin, betacellulin

and epiregulin (Toyoda et al., 1995; Van der et al., 1994). This domain also mediates the homodimerization of EGFR and the heterodimerization of EGFR with other members of EGFR family (Burden and Yarden, 1997; Van der, et al., 1994). The transmembrane domain is a single 23 residue α-helical transmembrane peptide. This domain anchors the receptor on the plasma membrane. The juxtamembrane region of EGFR links the transmembrane domain to the kinase catalytic core. It has been shown to play an important role in regulatory functions. Several intrinsic sorting signals have been mapped to the EGFR juxtamembrane region (Hobert et al., 1997), including autonomous basolateral sorting determinants (Hobert et al., 1997; He et al., 2002), a lysosomal sorting signal (Kil et al., 1999; Kil and Carlin, 2000), and a nuclear localization signal (Lin et al., 2001). The intracellular cytoplasmic domain also contains a 250 amino acid conserved protein tyrosine kinases core followed by a 229 residues carboxy-terminal tail with multi-tyrosine residues (Van der et al., 1994). The tyrosine kinase domain auto-phosphorylates tyrosine residues, which provides the phosphorylated tyrosine docking sites for downstream proteins. The carboxy-terminal regulatory domain becomes heavily phosphorylated and exerts both positive and negative effects upon receptor activation (Hunter and Lindberg, 1994).

Binding of ligands to EGFR at the plasma membrane induces dimerization of EGFR, which results in the activation of EGFR tyrosine kinase followed by receptor trans-autophosphorylation (Schlessinger and Ullrich, 1992). Downstream signaling proteins bind to sites of autophosphorylated tyrosine residues in activated EGFR to form receptor-signaling protein complexes, which then initiates the activation of

various signaling pathways (Carpenter, 1987) (Figure 1.3). Previous publications have shown that EGFR activation induces cell movement (Xie et al., 1998; Chen et al., 1994). Cell movement elicited by EGFR requires EGFR kinase activation and autophosphorylation (Chen et al., 1994b). At least two signaling pathways downstream of EGFR have been linked to cell movement: PLC-γ1 has been implicated in the pathway responsible for the reorganization of the cytoskeleton (Wells et al., 2002). Alternatively, EGFR activation leads to membrane ruffling and reorganization of focal adhesions through activation of member of the Rho subfamily of GTP-binding proteins (Ridley et al., 1992b; Ridley and Hall, 1992b).

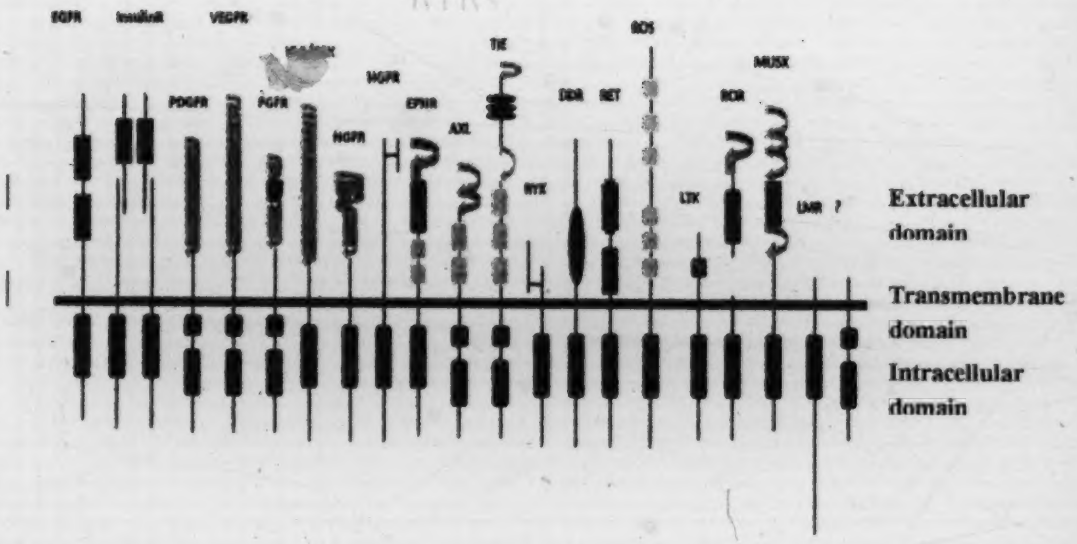


Figure 1.1 RTKs domain structure. The 20 human RTK classes all consist of extracellular (EX), transmembrane (TM), and intracellular domains. We can observe that the extracellular region which is very different between all the RTKs, and, on the other hand, that the intracellular region is very conserved.

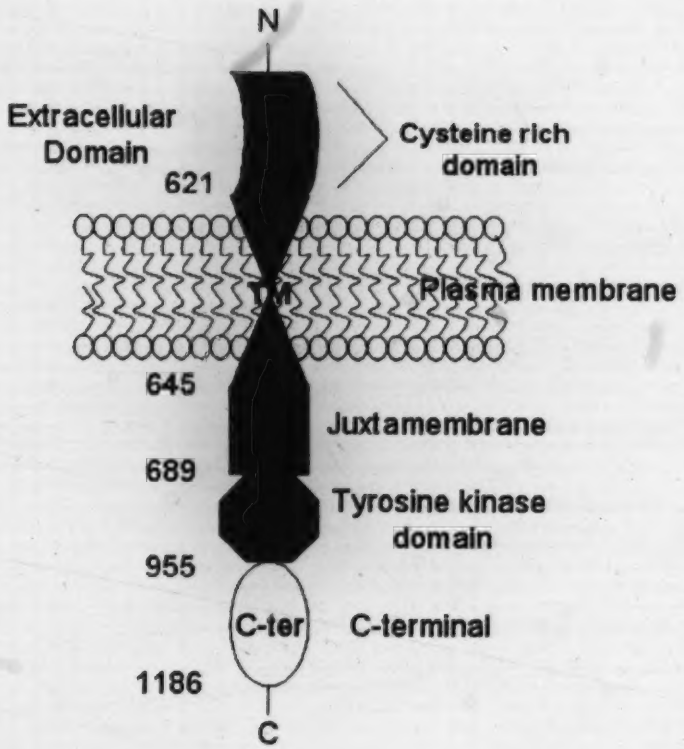


Figure 1.2 EGFR domain structure. Location of extracellular (EX), transmembrane (TM), juxtamembrane (JX), tyrosine kinase (TK), and carboxy-terminal (C-ter) domains in EGFR are labeled with the residue numbers indicating domain boundaries.

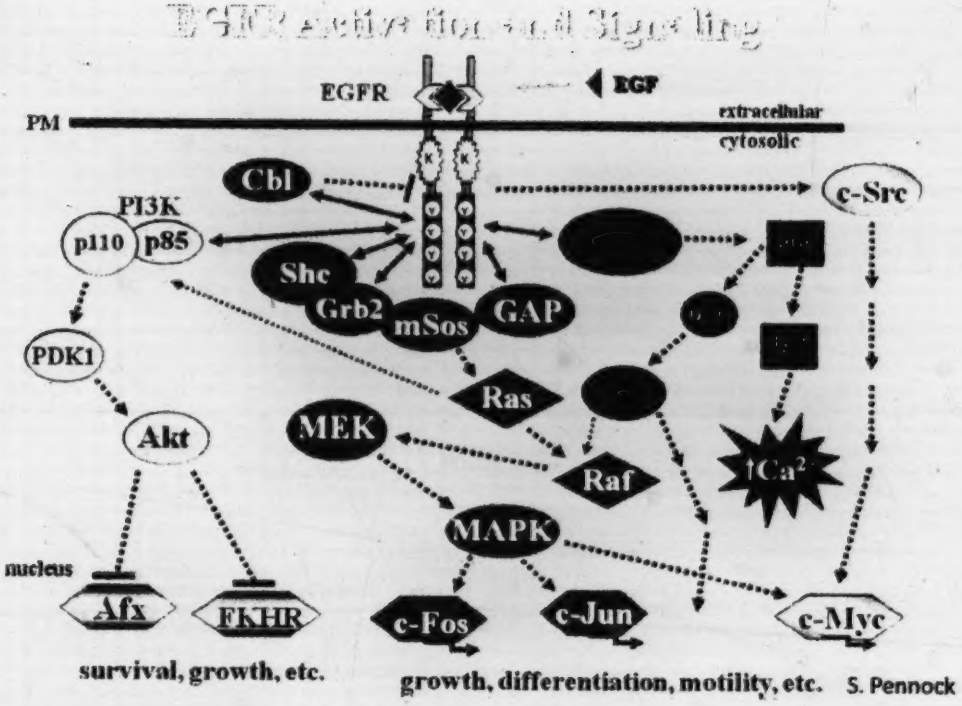


Figure 1.3 EGFR pathways. Upon EGF stimulation, EGFRs dimerize and then get trans-autophosphorylated. The dimerization and phosphorylation of EGFRs result in activation of downstream effectors for various pathways. PLC-γ1, c-Src, PI3K, GAP, Shc, Grb2 and mSos were listed here as examples.

1.3 PLC-γ1

PLC-γ1, a 145-kDa protein, is mainly present in cytosol. PLC-γ1 catalyzes the hydrolysis of phosphatidylinositol-4, 5-bis-phosphate (PtdIns 4, 5-P_2), creating inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG), and each of these products, described below, activates other proteins that play crucial roles in signaling inside the cell. IP_3 stimulates release of Ca^{2+} from internal stores, thereby mediating a variety of cellular processes, including fertilization and cell growth. DAG is an activator of Protein kinase C (PKC), a serine/threonine kinase involved in a wide range of cellular activities, including responses to hormones, neurotransmitters and growth factors (Thackeray et al., 1998).

PLC-γ1 contains two SH2 domains, one SH3 domain, two PH domains and X and Y catalytic domains (Figure 1.4). It forms a complex *in vivo* with activated EGFR through its SH2 domain interaction (Pawson, 1995; Margolis et al., 1990; Meisenhelder et al., 1989; Anderson et al., 1990). Complex formation leads to the phosphorylation of PLC-γ1 on tyrosine residues and an increase in its enzymatic hydrolysis activity, which is performed by X and Y catalytic domains (Kim et al., 1991; Ronnstrand et al., 1992; Rotin et al., 1992). All of the PLC-γ1 domains have been implicated in regulating the cellular localization of PLC-γ1 and in regulating growth factor (GF)-induced cell signaling. For example, both PLC-γ1 SH2 and PH domains have been involved in regulating GF-induced translocation of PLC-γ1 (Matsuda et al., 2001). We showed that the PH domain was not required for the initial translocation of PLC-γ1 from cytosol to the plasma membrane; however, it stabilizes

PLC-γ1 in membrane at a later time, and the PLC-γ1 SH2 and PH domains coordinate to determine EGF-induced translocation and activation of PLC-γ1 (Wang and Wang, 2003). PLC-γ1 SH3 domain has been implicated in directing cellular localization of the protein and targeting PLC-γ1 to cell skeleton (Bar-Sagi et al., 1993). PLC-γ1 SH3 domain is able to bind dynamin and Sos *in vitro* (Gout et al., 1993; Seedorf et al., 1994).

Recent studies have shown that PLC-γ1 is involved in much broader cell signaling than previously revealed. Interestingly, most recently identified interactions between PLC-γ1 and its binding proteins are mediated by its SH3 domain. The SH3 domain has a characteristic fold which consists of five or six beta-strands arranged as two tightly packed anti-parallel beta sheets. The linker regions may contain short helices (Yuzawa et al., 2004). The surface of the SH3-domain bears a flat, hydrophobic ligand-binding pocket which consists of three shallow grooves defined by conservative aromatic residues in which the ligand adopts an extended left-handed helical arrangement. The ligand binds with low affinity for each binding pocket but this may be enhanced by multiple interactions. The region bound by the SH3 domain is in all cases proline-rich and contains PXXP as a core-conserved binding motif (Pawson and Gish, 1992). Recently, it was shown that EGF stimulates the interaction between PLC-γ1 and phospholipase D2 (PLD_2) to potentiate EGF-induced IP_3 formation and Ca^{2+} increase. The interaction between PLC-γ1 and PLD_2 is mediated by PLC-γ1 SH3 domain and the proline-rich motif within the Phox homology (PX) domain of PLD2 (Jang et al., 2003). PLC-γ1 is essential for the activation of calcium entry into cells

after stimulation on cell-surface receptors and PLC-γ1 SH3 domain was required for this effect (Patterson et al., 2002). We recently showed that PLC-γ1 binds directly to protein kinase B (PKB/Akt) in response to EGF. The PLC-γ1-Akt interaction results in the serine phosphorylation of PLC-γ1 (Wang et al., 2006). One very interesting finding reported recently is that PLC-γ1 SH3 domain acts as a guanine nucleotide exchange factor (GEF) for PIKE and dynamin-1 (Ye et al., 2002; Choi et al., 2004). PLC-γ1 SH3 domain acts as a GEF for PIKE to regulate NGF-induced cell mitogenesis (Ye et al., 2002). PLC-γ1 SH3 domain acts as a GEF for dynamin-1 to regulate EGFR endocytosis and the interaction between PLCγ-1 SH3 domain and dynamin-1 is EGF-dependent (Choi et al., 2004).

PLC-γ1 is essential for cell motility induced by many growth factors, including EGF (Chen et al., 1994b), PDGF (Kundra et al., 1994), and HGF (Gual et al., 2000; Derman et al., 1996). While the mechanisms by which PLC-γ1 regulate cell motility is not clear yet, it was suggested that activation of PLC-γ1 by EGF resulted in the reorganization of cytoskeleton (Wells et al, 2002). PLC-γ1 hydrolyzes PIP_2 which leads to release of cofilin (Yonezawa et al., 1991; Yonezawa et al., 1990), gelsolin (Allen, 2003; Chou et al., 2002; Sun et al., 1999; Yonezawa et al., 1990) and profilin (Goldschmidt-Clermont et al., 1992; Goldschmidt-Clermont et al., 1991), and up on release these PIP_2 binding proteins they then locally remodel the actin cytoskeleton (Frantisek Baluska et al., 2001). The role of PLC-γ1 in the regulation of cell motility has been shown in a variety of cell types, especially carcinoma cells (Kassis et al., 2002). However, the exact role of how PLC-γ1 regulates cell motility is unclear.

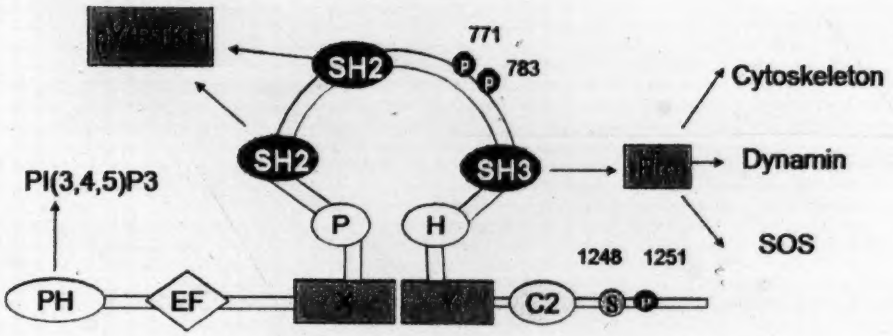


Figure 1.4 Domain structures of PLC-γ1. PLC-γ1 contains two SH2 domains, one SH3 domain, two PH domains and X and Y catalytic domains. SH2 domains binds to Y phosphorylated RTKs after receptor activation, which recruit PLC-γ1 from cytosol to plasma membrane. PH domains bind to phosphoinositrols with higher affinity and sustain PLC-γ1 on the membrane after its recruitment. XY domains are the catalytic domains. EF-hand motif contains helix-loop-helix structure and this motif appears capable of binding calcium or magnesium ions (reviewed in Rebecchi and Pentyala, 2000).

1.4 Rho GTPases family

Rho GTPases comprise a large subfamily of the Ras superfamily and include Cdc42, Rac and Rho proteins. Rho GTPases are guanine nucleotide binding proteins that cycle between an active, GTP-bound and an inactive, GDP-bound state. By cycling between inactive and active forms, Rho GTPases act as molecular switches to regulate signal transduction pathways that control a wide range of biological processes (Figure 1.5). For instance, Rho proteins are able to induce the reorganization of the actin cytoskeleton (Malliri and Collard, 2003). Since actin cytoskeletal changes are required for migratory behavior of cells in response to GF stimulation or matrix interactions (Turner C. E., 2000), Rho proteins play significant role in regulating cell migration and invasion of tumor cells. In fact, all aspects of cellular motility and invasion, including cellular polarity, cytoskeletal organization, and transduction of signals from the outside environment are controlled through an interplay between the Rho-GTPases (Sahai and Marshall, 2002; Lin and Van Golen, 2004). It has been shown that Cdc42 regulates the polarity of cell migration. Rac regulates the formation of membrane protrusions at the leading edge of migrating cells, which is required for forward movement. RhoA is required for the generation of contractile force leading to rounding of the cell body (reviewed in Etienne-Manneville and Hall, 2002; Malliri and Collard, 2003). In addition to their effects on cytoskeletal remodeling, Rho GTPases (Rac and Cdc42) stimulate the activity of the c-Jun N-terminal/stress-activated mitogen-activated protein kinase (Coso et al., 1995; Minden et al., 1995; Olson et al., 1995), and the p38/HOG mitogen-activated protein kinase (Zhang et al., 1995;

Bagrodia et al., 1995); are required (Rho) for activation of the serum response transcription factor, SRF (Hill et al., 1995); and induce (Rho, Rac, and Cdc42) G_1 cell cycle progression and DNA synthesis (Olson et al., 1995). Rho GTPases are also key components of signaling pathways leading to oncogenesis (Khosravi-Far et al., 1995; Qiu et al., 1995)

The activity of Rho proteins is tightly regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and GDP dissociation inhibitors (GDIs). GEFs activate Rho GTPases by exchanging bound GDP for GTP; GAPs stimulate GTP hydrolysis and thereby inactivate Rho GTPases, while GDIs prevent GDP dissociation from the small GTPases and thus inhibit their activation by GEFs. There are more than 60 potential GEFs and over 70 GAPs have been identified within mammalian genomes, and cell surface receptors may use different GEFs to activate specific GTPases (reviewed in Etienne-Manneville and Hall, 2002; Schmidt and Hall, 2002).

The prevailing cell biology literature has established that Rac family members stimulate formation of lamellipodia and membrane ruffles in cultured fibroblasts (Ridley et al., 1992; Ridley and Hall, 1992a; Burridge and Wennerberg, 2004; Ridley, 2001a; Ridley, 2001b). There are three different human Rac GTPase cDNAs described. Rac1 and Rac3 are expressed widely whereas Rac2 is a hematopoietic lineage-specific GTPase that regulates superoxide generation via the NADPH oxidase in phagocytes. Rac1 is a 21.4 kDa monomeric G protein mainly present in plasma membrane. It contains a common G-domain fold, which consists of a six-stranded β-sheet

surrounded by α-helices (Vetter and Wittinghofer, 2001). The differences between GDP and GTP bound forms are confined primarily to two segments, referred to as switch I and switch II regions (Milburn and Jeffrey, 1990). The amino acid sequence for Rac1 is shown in Figure 1.6. Rac1 is activated at the leading edge of motile cells and induces the formation of actin-rich lamellipodia protrusions, which serve as a major driving force of cell movement (Nobes and Hall, 1999; Kraynov et al., 2000; Small et al., 2002). The major downstream proteins that mediate actin polymerization in lamellipodia protrusions are the WAVE family proteins - the activators of the Arp2/3 complex (Miki et al., 1998; Yamazaki et al., 2003). Activated Arp2/3 complex induces rapid polymerization of actin and the formation of the branched actin filaments in lamellipodia (Welch and Mullins, 2002; Pollard and Borisy, 2003). However, precise mechanisms that lead to Rac1 activation during cell migration are not fully understood.

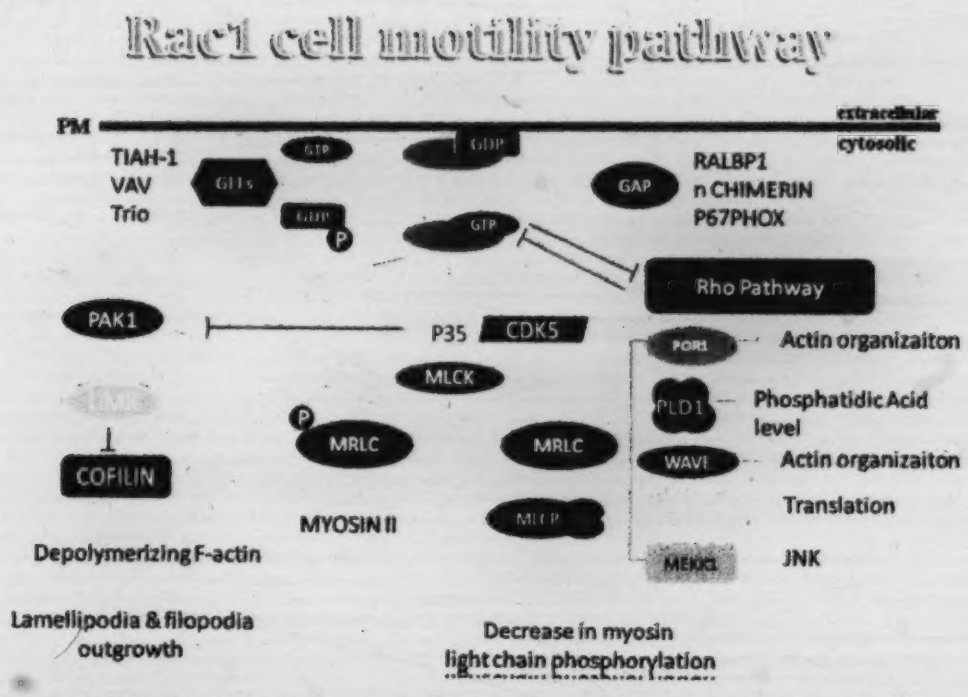


Figure 1.5 Rac1 cell motility pathways. Rac1 is a small G-protein in the Rho family that regulates cell motility in response to extracellular signals. Several changes in cytoskeletal structure and other aspects of cell structure are involved in cell motility. Rac1 is activated by GEF factors, and repressed by GAPs. GEFs are guanine nucleotide exchange factors, including Trio and Vav. Sos1 is involved in Ras signaling and also acts as a GEF for Rac to transduce signals between Ras and Rac. SWAP-70 is a Rac1 GEF that binds IP3 and transduces signals from tyrosine kinases to Rac1 to modulate the cytoskeleton and cause membrane ruffling. GAPs are GTPase- activating proteins. Rac1 stimulates the formation of actin-based structures such as filopodia and lamellopodia, while GAPs such as chimerin oppose the formation of these Rac dependent structures. Several different factors downstream of Rac1 act on cytoskeletal structure and other aspects of cell motility. Pak1 provides a direct link from Rac1 to cell motility through phosphorylation of the myosin light chain. Pak1 also phosphorylates and activates LIM kinase, which phosphorylates cofilin as one target. Cofilin stimulates actin depolymerization and changes in cell structure, and phosphorylation of cofilin by LIM kinase represses its activity. In neurons, Rac1 acts through the protein kinase cdk5 and p35 to phosphorylate and downregulate Pak1, increasing neuronal migration. Rac1 also interacts with several other factors to regulate a variety of processes. Interaction of Por1 with Rac1 is involved in membrane ruffling. WAVE is a member of the WASP family of proteins that regulate actin organization and that is involved in Rac1 signaling to cause membrane ruffling. Interaction of Rac1 with MEKK1 integrates Rac signaling with pathways signaling through map kinases. Cited from www.Biocarta.com

WT Rac1

MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPV

NLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRH

HC**PNTP**IILVGTKLDLRDDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLE

CSALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCLLL

Figure 1.6 Full amino acid sequences for Rac 1. The underlined region indicates the proline rich region.

1.5 Rationales, Hypothesis and Objectives

Given that both PLC-γ1 and Rho GTPases control cell motility by regulating the reorganization of cytoskeleton in response to EGF, it would be interesting to examine whether there is a direct functional linkage between PLC-γ1 and Rho GTPase in cell motility induced by EGF. Recently, it was shown that PLC-γ1 SH3 domain is a GEF for GTPases including Dynamin and PIKE (Ye et al., 2002; Choi et al., 2004). Thus, it is possible that PLC-γ1 may regulate cell motility and cancer metastasis by regulating Rho GTPase activity through its SH3 domain GEF activity.

My hypothesis is that the **SH3 domain of PLC-γ1 serves as a GEF for Rac 1 to regulate cell motility after EGF stimulation.**

In this research we investigated the interaction between PLC-γ1 and Rac1 as well as the GEF activity of PLC-γ1 on Rac1, both *in vitro* and *in vivo*.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals, enzymes and kits were used according to the manufacturers' specifications and in accordance with protocols set out by the Environmental Health and Safety of the University of Alberta and Workplace Hazardous Materials Information System (WHMIS).

2.1.1 Chemicals and Reagents

Acetic acid, glacial	BDH
Acrylamide/bis	Biorad
Agar	Gibco
Agarose	Gibco
Ammonium persulfate	BDH
Aprotinin	Sigma
Bacto-tryptone	Difco
β-mercaptoethanol	Sigma
Bromodeoxy uridine (BrdU)	Amersham
Bromophenol blue	Biorad
Buria-Bertani media, broth base	Gibco
Calcium chloride	Sigma
Coomasie brilliant blue, G250	Biorad
Dimethyl sulfoxide	Fisher
Disulfosuccinimidyl suberate (DSS)	Calbiochem
Dulbecco's modified eagle medium (DMEM)	Gibco
EGF	Upstate
Epidermal growth factor	Upstate
Ethanol, 95%	Fisher
Ethidium bromide	OmniPur
Ethylene glycol-bis (b-amino ethyl ether tetra acetic acid) (EGTA)	Sigma
Fetal bovine serum	Sigma
Glucose	EM science
Glycerol	BDH
Glycine	Biorad
Hydrochloric acid	Fisher
Isopropanol	Fisher
Kanamycin	Sigma
Magnesium chloride	BDH

Manganese chloride	Fisher
Monensin	Calbiochem
N,N-bis [2hydroxyethyl]-2-aminoethanesulfonic acid (BES)	Sigma
Nonidet P40	BDH
Pepstatin a	Sigma
Phosphate buffered saline, 10x	OmniPur
PIPES	Sigma
Potassium chloride	BDH
Sodium chloride	BDH
Sodium azide	Sigma
Sodium dodecyl sulfate (SDS)	Biorad
Sodium fluoride	Sigma
Sodium orthovanadate	Sigma
Sucrose	Biobasic
Tetramethylethylenediamine(TEMED)	Gibco
Tris(hydroxymethyl) aminomethane	Biorad
Triton X-100	BDH
Tween 20	Fisher
Yeast extract, select	Gibco

2.1.2 Other Materials

Medical X-ray Film	Fuji
Transblot Nitrocellulose	Biorad
Whatman Chromatography Paper	Fisher
Glutathione insolubilized on cross-linked 4% agarose	Sigma
Goat anti-mouse IgG conjugated with agarose	Sigma
Protein A sepharose 6MB	Pharmacia

2.1.3 Enzymes

DNA ligase, T4	Gibco
Hot Start Taq, DNA polymerase	Qiagen
dNTPs	Invitrogen
Restriction endonucleases	Gibco
RNAase	Sigma

2.1.4 Experimental Kits

QIAprep Spin Mniniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
HiSpeed Plasmid Midi Kit	Qiagen
Supersignal ECL Western Blotting Detection Reagents	Pierce
Topo-XL PCR cloning kit	Invitrogen
RhoGEF Exchange Assay Kit	Cytoskeleton

2.1.5 Plasmids

pCR-XL-TOPO	Invitrogen
pEGFP-N3	Clontech
pEYFP-C1	Clontech
pGEX-3X	Clontech
pEGFP-C3	Clontech

2.1.6 Antibodies

2.1.6.1 Primary Antibodies

Mouse anti-phospho-PLC-γ1 (Tyr 783)	Medicore
Mouse anti-phospho-Tyrosine (pY99)	Santa Cruz
Mouse anti- PLC-γ1	Upstate
Mouse anti-tubulin	Santa Cruz
Rabbit anti-GFP	Clonetech
Rabbit anti-phosphoserine (1248) of PLC-γ1	Upstate
Rabbit anti-phospho- PLC-γ1 (Tyr 783)	Santa Cruz
Rabbit anti- PLC-γ1	Santa Cruz
Mouse anti-Rac 1 mono clone	Upstate

2.1.6.2 Secondary Antibodies

FITC-conjugated anti-mouse	Jackson ImmunoResearch
FITC-conjugated anti-Rabbit	Jackson ImmunoResearch
Rhodamin-conjugated anti-mouse	Jackson ImmunoResearch
Rhodamin-conjugated anti-Rabbit	Jackson ImmunoResearch
Horseradish peroxidase(HRP)- conjugated anti-rabbit	Bio-Rad
Horseradish peroxidase(HRP)- conjugated anti-mouse	Bio-Rad

2.1.7 Molecular Size Markers

100 bp DNA ladder	Gibco

1 kb DNA ladder	Gibco
Prestained protein markers for SDS-PAGE	Sigma

2.1.8 Buffers and Other Solutions

A list of all buffers and solutions used in this study is provided in Table 2.1

Table 2.1 Buffers and other solutions used in this study

Solution	Composition
Acidic Stripping buffer	100 mM acetic acid, 150 mM NaCl, pH 2.7
BOS buffer	50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% NP-40, 10% glycerol, 10 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA
GST wash buffer	20 mM HEPES pH 7.5, 120 mM NaCl, 10% glycerol, 0.5% NP-40, 2 mM EDTA
Homogenization buffer	20 mM Tris-HCl, pH 7.0, 1 mM $MgCl_2$, 4 mM NaF
Immunoprecipitation buffer	20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) NP40, 0.1% (w/v) sodium deoxycholate, 100 mM NaF and 5 mM $MgCl_2$
Phosphate-buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer
Protease inhibitor cocktail	0.5 mM Na_3VO_4, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin and 1 μM pepstatin A
SDS-loading buffer	250 mM Tris-HCl, 40% (v/v) glycerol, 8% (w/v) sodium dodecyl sulfate, 20% (v/v) b-mercaptoethanol, 2% (w/v) bromophenol blue
SOC medium	2% bacto typtone, 0.5% yeast extract, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
Transfer buffer	48 mM Tris-HCl, 39 mM Glycine, 20% (v/v)

	methanol, 0.03% (w/v) sodium dodecyl sulfate
Transformation buffer	10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl
Triton X-100 lysis buffer	0.4% triton X-100, 140mM NaCl, 50mM Tris-HCl, pH 7.2, 1 mM EGTA
GST-beads buffer	50 mN Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT)
Factor Xa cleavage buffer	50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$
GST-PAK buffer	50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$

2.1.9 Oligonucleotides

A list of all oligonucleotides and their manufacturers is included in Table 2.2

Table 2.2 Oligonucleotides used in this study

Name	Sequence	Manufacturer
Rac 1 P/P PM Leading	CGG CAC CAC TGT GCC AAC ACT GCC ATC ATC CTA GTG	Sigma
Rac 1 P/P PM Lagging	CAC TAG GAT GAT GGC AGT GTT GGC ACA GTG GTG CCG	Sigma
Rac 1 N17 Leading	GGA GCT GTA GGT AAA AAT TGC CTA CTG ATC AG	Sigma
Rac 1 N17 Lagging	CT GAT CAG TAG GCA ATT TTT ACC TAC AGC TCC	Sigma
Rac 1 L61 Leading	GG GAT ACA GCT GGA CTA GAA GAT TAT GAC AG	Sigma
Rac 1 L61 Lagging	CT GTC ATA ATC TTC TAG TCC AGC TGT ATC CC	Sigma

PLC - γ1- SH3 P842L Leading	G CAG CTG TGG TTC CTA TCA AAC TAC GTG G	Sigma
PLC - γ1- SH3 P842L Lagging	C CAC GTA GTT TGA TAG GAA CCA CAG CTG C	Sigma

2.2 Methods

2.2.1 *Cell culture, transfection, and treatment*

COS-7 cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to transfection, COS-7 cells were seeded into 100 mm plates and incubated until cells are 40-60% confluency. The transfections were performed using Lipofectin Reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The transfection efficiency of COS-7 cells for all experiments using Lipofectin Reagent was 60% to 70%. For the EGF treatments, COS-7 cells were serum starved for 12 h, and EGF was added to a final concentration of 100 ng/ml.

2.2.2 *Plasmids*:

The YFP-tagged full-length and SH3 domain deletion mutant of PLC-γ1 were generated previously (Wang and Wang, 2003) (Figure 2.1). Various GST fusion proteins (including PLC-γ1-N-SH2, PLC-γ1-C-SH2, PLC-γ1-SH3, PLC-γ1-SH3 P842L, and PLC-γ1-N-PH) were generated previously in the laboratory (Wang et al., 1998). GST-PAK fusion proteins were gift from Dr. Gary Eitzen (University of

Alberta). GFP-Rac 1 was a gift from Dr. Mark R. Philips (NYU School of medicine). All the mutants with point mutation were created with the QuikChange multiple site-directed mutagenesis kit (Stratagene, La Jolla, CA) with GFP-tagged wild type Rac1 (wt Rac1) as template. These mutants include a GFP-tagged mutant Rac1 with mutation T17 to asparagine (termed N17), a GFP-tagged mutant with mutation of Q61 to leucine (termed L61), and a mutant of GFP-tagged Rac1 with mutations of both P106 and P109 to alanine (termed Rac1 PP/AA).

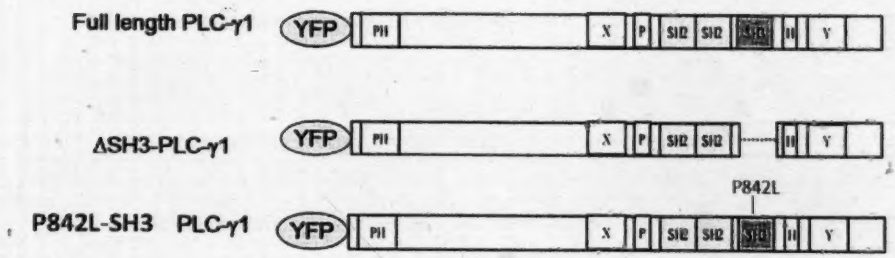


Figure 2.1 Wild-type, P842L SH3 domain and ΔSH3 domain mutants of PLC-γ1. All PLC-γ1 mutants are tagged with YFP. ΔSH3-PLC-γ1 is the YFP tagged full length PLC-γ1 with SH3 deletion; and the P842L-SH3 PLC-γ1 is the YFP tagged full length PLC-γ1 with a single point mutation of P842L in SH3 domain.

2.2.3 *Expression and Purification of GST Fusion PLC-γ1 Domains*

To purify N-SH2, C-SH2, SH3 domains and P842L SH3 domain of PLC-γ1, the pGEX-3X plasmid containing the wild-type N-SH2, C-SH2, SH3, and P842L SH3 domains of PLC-γ1 were transformed into *Escherichia coli* DH5α. Bacteria were grown to an optical density $(OD)_{600}$ of 0.3-0.4 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h at 37 °C. After pelleting, bacterial cells were lysed by sonication in GST-beads buffer containing 50 mN Tris, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), containing protease inhibitors [0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A]. Triton X-100 was added to a final concentration of 1% (v/v), and particulates were removed by centrifugation for 10 min at 10,000 rpm in a JA-17 (Beckman Coulter, Fullerton, CA) rotor. The clarified lysate was incubated with glutathione-agarose beads (Sigma-Aldrich) for 1 h at 4 °C, washed three times with ice-cold GST-wash buffer containing 1 mM DTT plus protease inhibitors and protein bound to the glutathione-agarose beads were stored at 4 °C.

To purify PH domain of PLC-γ1, *E. coli* BL-21, a strain that is defective in OmpT and Lon protease production and expresses the fusion protein in a more soluble and intact form, were used for transformation. Cells were grown to an OD_{600} of 0.3-0.4 and induced with 0.1 mM IPTG for 8-12 h at 22 °C. The later steps were the same as the other GST-mutants (Fig. 2.2).

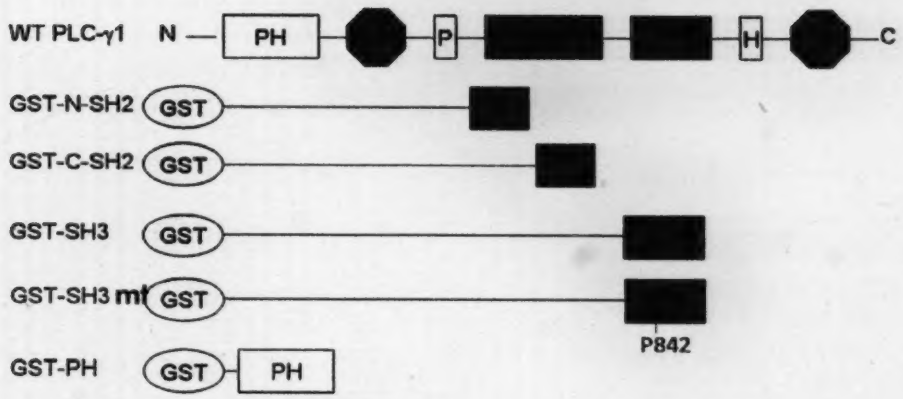


Figure 2.2 Stuctures of GST fusion PLC-γ1 domains. All domains were tagged with GST shown in blue in their N-terminals. Different individual PLC-γ1 domain that is fused with GST was labeled according to wt PLC-γ1's domain.

2.2.4 *in vitro Binding Assay*

COS-7 cells were treated with or without EGF and then lysed into BOS buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% NP-40, 10% (v/v) glycerol, 10 mM $MgCl_2$, and 1 mM EDTA) with protease inhibitors. The lysates were centrifuged at 21,000 X g at 4 ℃ for 30 min. Supernatants were used in the binding assay. GST fusion PLC-γ1 proteins bound to glutathione-agarose-beads in BOS buffer were added and incubated at 4 ℃ for 1 h. Beads were collected by centrifugation, washed three times with BOS buffer, and then loading buffer was added. The pull-down proteins were resolved on SDS-PAGE and analyzed by immunoblotting with anti-GST and anti-Rac1 antibodies.

Table 2.3 Antibodies and their dilutions used for Western Blotting

Antibody	Dilution	Manufactures
Mouse anti-PLC-γ1	1:1000	Upstate
Mouse anti-Rac 1 monoclonal	1:500	Upstate
Rabbit anti-GFP	1:5000	Clonetech
Rabbit anti-PLC-γ1	1:500	Santa Cruz
Horseradish peroxidase(HRP)- conjugated anti-rabbit	1:2000	Bio-Rad
Horseradish peroxidase(HRP)- conjugated anti-mouse	1:2000	Bio-Rad

2.2.5 *Immunoprecipitation*

Immunoprecipitation experiments were carried out as described previously (Wang et al., 1999). Briefly, cells were lysed with immunoprecipitation buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 100 mM NaF, 5 mM Mg_2Cl, 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A] for 2 h at 4 ℃ . Cell lysates were centrifuged at 22,000 X *g* for 30 min to remove debris. The supernatants, containing 1

mg of total protein were precleared with the agarose beads, and then were used to incubate with 1 μg of specific antibody for 2 h at 4 °C with gentle mixing by inverting. Then, goat anti-mouse IgG conjugated with agarose or protein A conjugated with agarose was added to each fraction and incubated for 2 h at 4 °C with agitation. Finally, both the agarose beads and the nonprecipitated supernatant were collected by centrifugation. For the controls, mouse or rabbit non-specific IgG were used instead of specific primary antibodies. The agarose beads were washed three times with immunoprecipitation buffer, and 1X loading buffer was added. The sample was boiled for 5 min and ready for SDS-PAGE followed by Western blot. Antibodies used for immunoprecipitation were listed in Table 2.4.

Table 2.4 Antibodies and their concentrations used for immunoprecipitation.

Antibody	Concentration(ng/ml)	Manufactures
Mouse anti-Rac1 mono clone antibody	500	Upstate
Mouse anti-PLC-γ1	500	Upstate
Rabbit anti-GFP	100	Clonetech
Goat anti-GFP	10	Gift from Dr. Luc Berthiaume

2.2.6 *Immunofluorescence*

Indirect immunofluorescence was carried out as described previously (Wang et al., 1999). Cells were grown on glass coverslips to subconfluence and serum starved for 24 h. After treatment with EGF (100 ng/ml) for the indicated time, the cells were fixed by immersion in -20 °C methanol or 4% of paraformaldehyde for 5 min and 15 min respectively. Upon removal of the methanol and washing with PBS, the cells were then

permeabilized with 0.2% Triton X-100 for 15 min at room temperature. The coverslips were incubated for 1 h at room temperature with the primary antibody, followed by 45 min incubation with second antibody, respectively. The stained cells were analyzed by conventional fluorescence microscopy and Zeiss (Carl Zeiss, Thornwood, NY) oil immersion lens. Color photographs were taken with a digital camera by superimposing the monochrome graphs of two channels. Antibodies and their concentrations were listed in Table 2.5.

Table 2.5 Antibodies and their dilutions used for Immunofluorescence

Antibody	Dilution	Manufactures
Rabbit anti-PLC-γ1	1:50	Santa Cruz
Mouse anti-Rac 1 mono clone	1:100	Upstate
FITC-conjugated anti-mouse	1:200	Jackson ImmunoResearch
Rhodamin-conjugated anti-Rabbit	1:200	Jackson ImmunoResearch
Rhodamin-Phalloidin	1:1000	Molecular Probes

2.2.7 *in vitro GEF activity assay*

To purify the SH3 domains of PLC-γ1, the pGEX-3X plasmid containing GST fused wild type SH3 domain was transformed into *Escherichia coli* DH5α. Bacteria were grown to an optical density $(OD)_{600}$ of 0.3-0.4 and induced with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h at 37 °C . After pelleting, bacterial cells were lysed by sonication in 50 mM Tris, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), containing protease inhibitors [0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A]. Triton X-100 was added to a final concentration of 1%, and particulates were removed by centrifugation for 10 min at 10,000 rpm in a JA-17 (Beckman

Coulter, Fullerton, CA) rotor. The clarified lysate was incubated with glutathione-agarose beads (Sigma-Aldrich) for 1 h at 4 °C , washed tree times with Factor Xa cleavage buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$. Factor Xa was added to 50% slurry beads with final concentration of 50 μg/ml followed by agitation for 16 h at room temperature to cleave SH3 domains from GST-SH3 immobilized on beads. The supernatant of beads were recovered and cleaved SH3 domains were visualized by coomassie blue staining.

In vitro GEF activity assay was then carried out according to instructions of RhoGEF EXCHANGE ASSAY BIOCHEM KIT (Cytoskeleton, Inc. Denver). Briefly, fluorescence spectroscopic analysis of *N*-methylanthraniloyl (mant) - GTP incorporation into purified His-Rac1 was carried out using PTI QM-4 SE spectrometer at 22 °C. Exchange reaction assay mixtures containing 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 50 ug/ml bovine serum albumin (BSA), 0.75 μM mant-GTP, and 2 μM of Rac1 GTPases were prepared and allowed to equilibrate with slow continuous stirring. After equilibration, the mixtures were placed into 4 sample holders and fluorescence measurements were taken approximately every second with excitation and emission wavelengths of 360 nm and 440 nm respectively and 10 nm bandwidth. After 200 readings (200 seconds), purified SH3 domain/Dbs/Water was added to 0.8 μM and the relative mant fluorescence (λ_{ex} = 360 nm, λ_{em} = 440 nm) was monitored.

2.2.8 *in vivo GEF activity assay:*

COS-7 cells with or without various types of transfections were grown to 40-60%

confluence before transfection. YFP-tagged full-length and SH3 domain deletion mutant of PLC-γ1 DNA were transfected using Lipofectin Reagent into cells according to the manufacturer's instructions. Then COS-7 cells were serum starved for 12 h followed by EGF (100 ng/ml) stimulation for different time period. The cells were lysed into GST-PAK buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$) with protease inhibitors. The lysates were centrifuged at 21,000 X g at 4 °C for 15 min. Supernatants were used in the binding assay. GST-PAK fusion proteins bound to glutathione-agarose-beads in GST-PAK buffer were added and incubated at 4 °C for 1 h. Beads were collected by centrifugation, washed three times with GST-PAK buffer, and then SDS loading buffer was added. The samples were resolved on SDS-PAGE and analyzed by immunoblotting with anti-GST and anti-Rac1 antibodies. For the GTP-γS positive control, COS-7 cells were lysed in GST-PAK buffer and lysates were centrifuged at 21,000 X g at 4 °C for 15 min. Supernatants were collected. EDTA and GTP-γS were added to cell lysates with final concentration of 10 mM and 100 μM respectively. The mixture then was incubated at 30 °C for 15 min followed by adding $MgCl_2$ to final 60 mM. The lysates were then used for GST-PAK binding assay.

2.2.9 *Wound Healing Assay*

COS-7 cells were grown on 24-well plate at 40–60% confluence and transfected with different GFP-tagged PLC-γ1 constructs. After 24–48 h, the cells reached 100% confluence, and subjected to serum starvation for 24 h to synchronize cells to G1 phase.

A wound was created with a glass pipette after serum starvation and a nearby reference point was created by a needle. The plate was washed once with serum free medium and replaced with desired medium. The cells were observed under fluorescence microscope to ensure that enough cells in the leading edge of the wound were positively transfected. Both phase contrast and fluorescence images were acquired every 2 h by matching the reference point until the wound has completely closed. To calculate the rate of migration of the transfected cells, we measured the distance traveled toward the center of the wound after 8 h. We performed wound healing assay on G1 phase synchronized COS-7 cells only for 8 h with EGF stimulation, which can limit the effect of the DNA synthesis in S phase. At least eight to 10 randomly chosen areas including at least 50 cells were quantified. Experiments were repeated three to four times, and an individual photograph is chosen as the example. The relative distance to the reference line is calculated and normalized to the non-transfected cells (Endo), and data are expressed as means $\pm$ SEs of the percentage of the non-transfected cells from at least 8 to 10 randomly chosen areas from three to four separate experiments. Significance of the set of data was represented using P-value generated from normal student t-tests.

Chapter 3

Results

3.1 Co-localization of PLC-γ1 and Rac1 at the plasma membrane in response to EGF.

As a portion of PLC-γ1 is translocated from cytosol to the plasma membrane and then to endosomes in response to EGF (Wang and Wang, 2003), we first determine the localization of Rac1 and trans-locolization of PLC-γ1 in response to EGF in COS-7 cells transfected with GFP tagged Rac1. The cells with transfected GFP-Rac1 were serum starved for 12 h followed by EGF (100 ng/ml) treatment for different time period. The localization of GFP-Rac1 was visualized by its intrinsic fluorescence and the localization of PLC-γ1 was visualized by indirect immunofluorescence (Fig. 3.1). In Figure 3.1, red color represents PLC-γ1 and green color represents GFP-Rac1. We showed that with or without EGF stimulation, Rac1 is localized mainly on the plasma membrane. Following EGF stimulation for 5 to 15 min, Rac1 is still localized to the plasma membrane but more concentrated to the membrane ruffling sites (data not shown). Meanwhile, we showed that PLC-γ1 localizes in the cytosol without EGF stimulation (Fig. 3.1). We also showed that PLC-γ1 and Rac1 began to co-localize on the plasma membrane following EGF stimulation for 5 min (Fig. 3.1). Following EGF stimulation for 15 min, a portion of PLC-γ1 was localized to endosomes, however, a significant amount of PLC-γ1 still colocalized with Rac1 at the plasma membrane (Fig. 3.1). The results were consistent with our previous findings. Rac1 localization on the plasma membrane with or without EGF is not affected by knocking out the endogenous PLC-γ1 by siRNA specific to PLC-γ1 (Fig 3.2). This indicates that PLC-γ1 does not mediate Rac1 membrane localization and co-locolization of PLC-γ1 and Rac1 is in a

PLC-γ1 translocation dependent manner. Furthermore, we tested the translocation ability of PLC-γ1 without SH3 domain (Fig 3.3). YFP-ΔSH3 PLC-γ1 was transfected into COS-7 cells and the endogenous Rac1 were visualized by anti-Rac1 antibodies using double indirect immunofluorescence. Figure 3.3 showed that without SH3 domain, PLC-γ1 can still translocate to plasma membrane upon receptor engagement and the co-localization with Rac1 is also not affected. This result indicated that SH3 domain does not affect PLC-γ1 tracslocation, thus PLC-γ1 with SH3 deletion also have chance to contact Rac1 on plasma membrane.

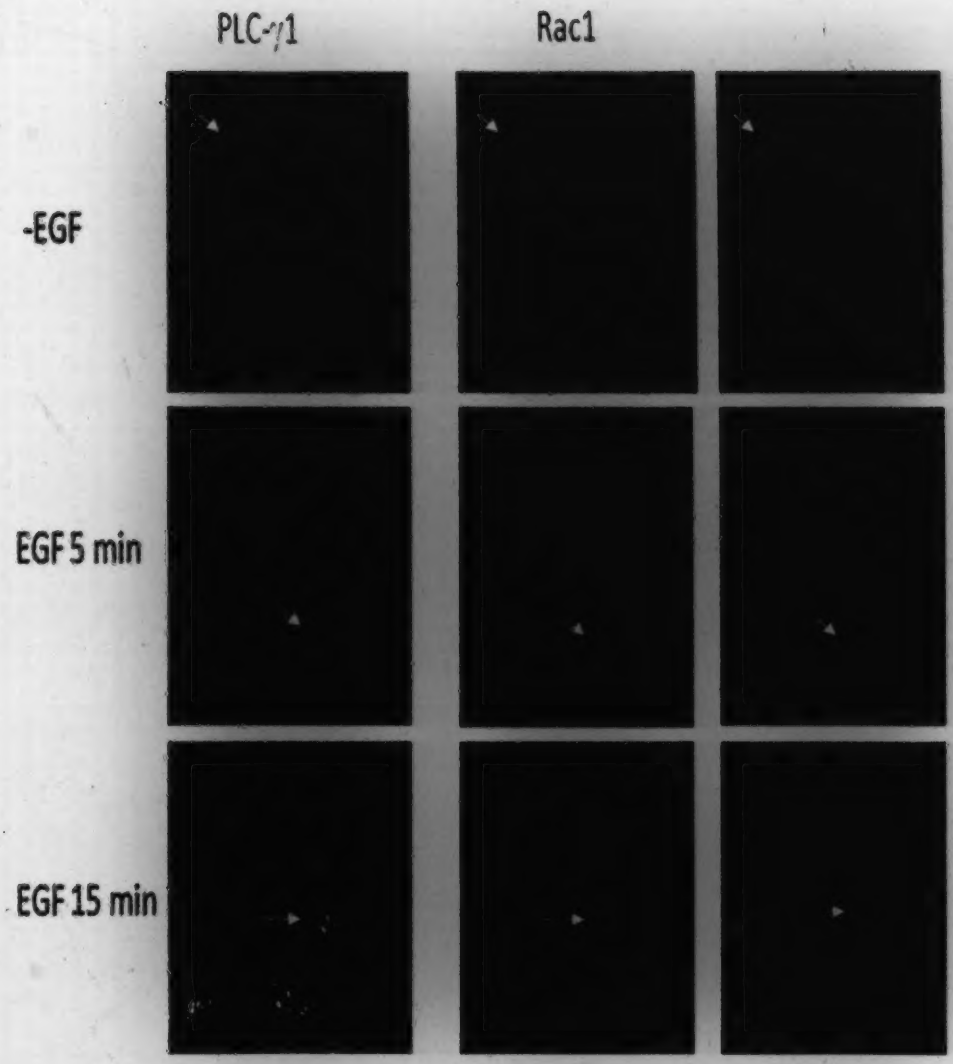


Figure 3.1 Co-localization of PLC-γ1 and Rac1 in COS-7 cells. Co-localization of endogenous PLC-γ1 and Rac1 by double indirect immunofluorescence. COS-7 cell treated or not treated with EGF as indicated. Colocalization (yellow) of Rac1 (Green) and PLC-γ1 (red) was indicated by arrow. Size bar = 15 μM.

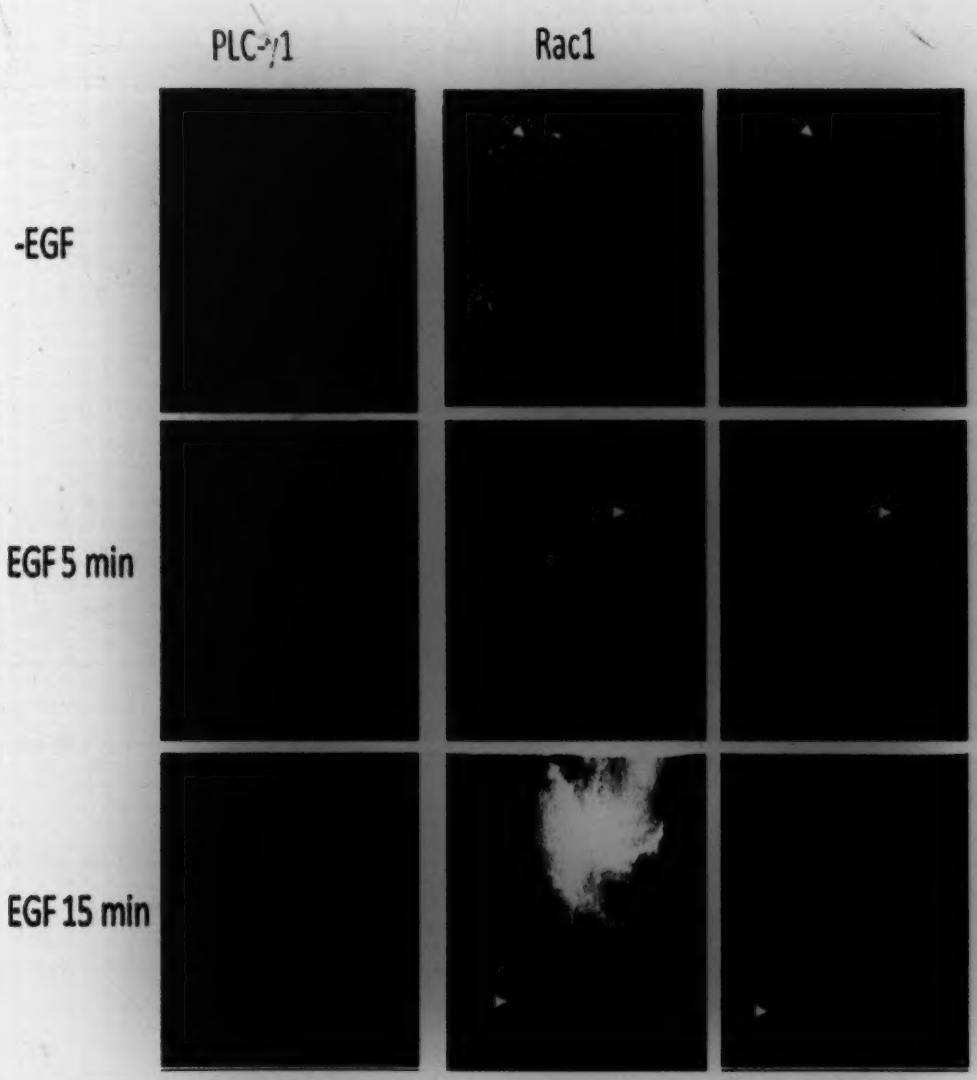


Figure 3.2 Lcalization of Rac1 and in COS-7 cells with PLC-γ1 knocking out. Localization of endogenous PLC-γ1 and Rac1 by double indirect immunofluorescence. COS-7 cells were transfected with siRNA specific to endogenous PLC-γ1 for 2 days and then serum starved for 12 h followed by treatment of EGF as indicated. Colocalization (yellow) of Rac1 (Green) and PLC-γ1 (red) was indicated by arrow. Size bar = 20 μM.

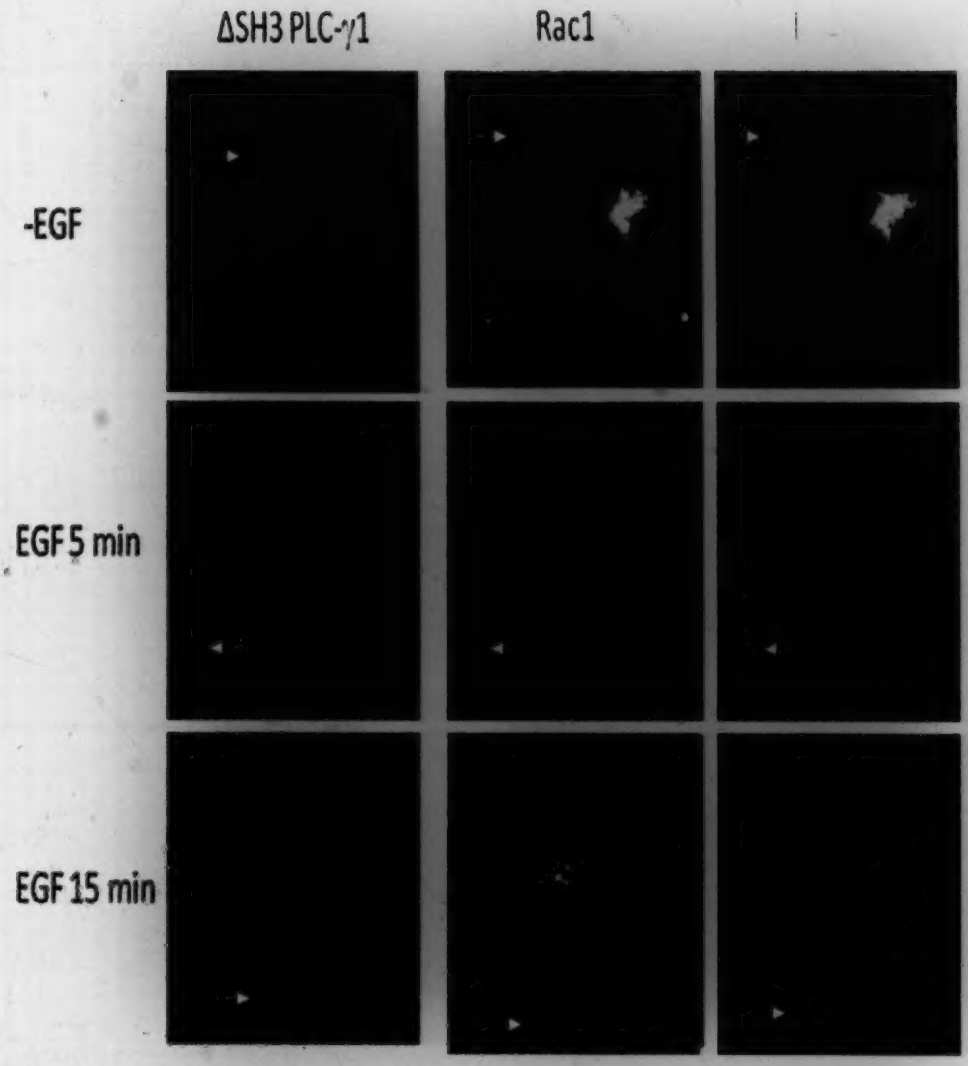
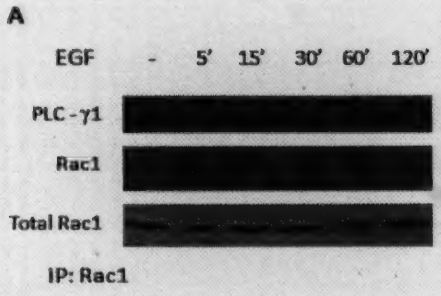


Figure 3.3 Localization of Rac1 and YFP-ΔSH3 PLC-γ1 in COS-7 cells. Localization of YFP-ΔSH3 PLC-γ1 and Rac1 by double indirect immunofluorescence. COS-7 cell treated or not treated with EGF as indicated. Colocalization (yellow) of Rac1 (Red) and YFP-ΔSH3 PLC-γ1 (Green) was indicated by arrow. Size bar = 20 μM.

3.2 Association between PLC-γ1 and Rac 1 in response to EGF

We next determined whether PLC-γ1 and Rac1 can physically associate in response to EGF. We examined the association by co-immunoprecipitation. COS-7 cells were treated with EGF for various times, and the Rac1 was immunoprecipitated with mouse anti-Rac1 antibody. The co-IP of PLC-γ1 was examined by immunoblotting with antibodies to both Rac1 and PLC-γ1. As shown in Figure. 3.4, equal amounts of Rac1 were precipitated for each condition and PLC-γ1 was co-immunoprecipitated with Rac1 following EGF stimulation. This interaction was reached maximum levels at 5 to 15 min. The strong interaction retained until 30 min after EGF stimulation and dissociation occured after that. The association between PLC-γ1 and Rac1 is consistent with their co-locolization pattern showed in Figure 3.4.



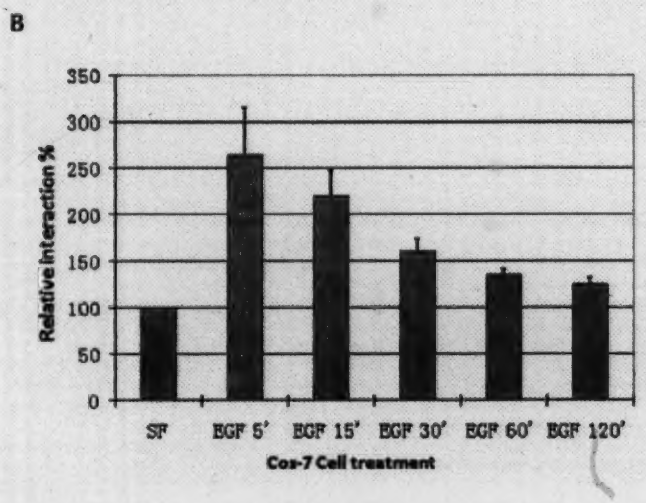


Figure 3.4 The EGF-induced association of PLC-γ1 and Rac1. COS-7 cells were treated with EGF (100ng/ml) for various times (unit: min), and the Rac1 was immunoprecipitated with mouse anti-Rac1 antibody. The co-immunoprecipitated proteins were then resolved in SDS-PAGE followed by immunoblotting with antibodies to both Rac1 and PLC-γ1. (A) Western blots showing the association between PLC-γ1 and Rac1. (B) Quantitative bar graph representing the average of 3 repeats.

3.3 PLC-γ1 SH3 domain mediates the association between PLC-γ1 and Rac1.

We next determined which domain of PLC-γ1 is responsible for binding to Rac1. PLC-γ1 contains two SH2 domains, one SH3 domain, one intact PH domain, and one split PH domain. We had previously fused each of these domains to GST (Wang and Wang, 2003; Wang et al., 1998). These GST fusion proteins were used to pull-down Rac1 from COS-7 cell lysates with or without EGF stimulation *in vitro*. As shown in Figure. 3.5A, with or without EGF stimulation only PLC-γ1 SH3 domain is able to pull down Rac1. This indicates that PLC-γ1 SH3 domain specifically interacts with Rac1. To further test whether the interaction between SH3 and Rac1 is direct and whether the SH3 domain interaction with Rac1 is specific, we generated a GST fusion with a loss of function mutant P842L SH3 and incubated P842L SH3 and the other GST fusion domains with purified Rac1 (Cytoskeleton Inc. Cat. # RC01) in pure buffer system. Figure 3.5B shows that only wt SH3 domain can interact with purified Rac1 *in vitro* but not the non-functional P842L SH3 domain. Consistently with our previous results, other domains cannot associate with Rac1 either *in vitro*. After all, the results suggested that SH3 domain of PLC-γ1 interact with Rac1 directly *in vitro*. It is interesting to note that the Rac1 amount of pulled down by GST-SH3 domains was similar in both conditions with or without EGF stimulation *in vitro*.

We next determined whether the PLC-γ1 SH3 domain mediates the interaction between PLC-γ1 and Rac1 *in vivo*. We transfected COS-7 cells with YFP-tagged full-length PLC-γ1, YFP-tagged PLC-γ1 containing P842L SH3 domain (P842L SH3 PLC-γ1) or YFP-tagged mutant PLC-γ1 lacking of the SH3 domain (PLC-γ1ΔSH3).

All the constructs were generated previously (Wang and Wang, 2003). Following the transfection, EGF-stimulated interaction between Rac1 and PLC-γ1 was examined by co-immunoprecipitation. Rac1 was immunoprecipitated by mouse anti-Rac1 antibody, and the co-immunopricipitation of YFP tagged PLC-γ1 and Rac1 were revealed by immunoblotting with anti-GFP and Rac1 antibodies. As expected, similar amount of Rac1 was precipitated for each condition. Wild type PLC-γ1 was strongly co-immunopricipitated with Rac1 after EGF treatment, which confirmed our early observation. However, both P842L SH3 PLC-γ1 and PLC-γ1ΔSH3 were not co-immunoprecipitated with Rac1 with or without EGF stimulation. This indicates that a functional SH3 domain is required for the interaction between PLC-γ1 and Rac1 (Fig. 3.6A). Additionally, we also immunoprecipitated the YFP-tagged PLC-γ1 and the mutants with anti-GFP antibodies and bound Rac1 was analyzed by immunoblotting. As shown in Figure 3.6B, Rac1 was co-immunoprecipitated only with wt PLC-γ1 after EGF stimulation but not with YFP-P842L SH3 PLC-γ1 or YFP-ΔSH3 PLC-γ1, which further indicates that PLC-γ1 SH3 domain is required for the EGF-induced interaction between PLC-γ1 and Rac1.

To determine whether PLC-γ1 only specifically interacts with Rac1 or broadly interact with other Rho proteins, we examined the interaction between PLC-γ1 and Cdc42 or RhoA by co-immunoprecipitation. The same GFP-PLC-γ1 immunoprecipitates prepared above were immunoblotted with antibodies to Cdc42 and RhoA. As shown in Figure. 3.6B, no Cdc42 and RhoA were detected. This indicates that PLC-γ1 specifically interacts with Rac1.

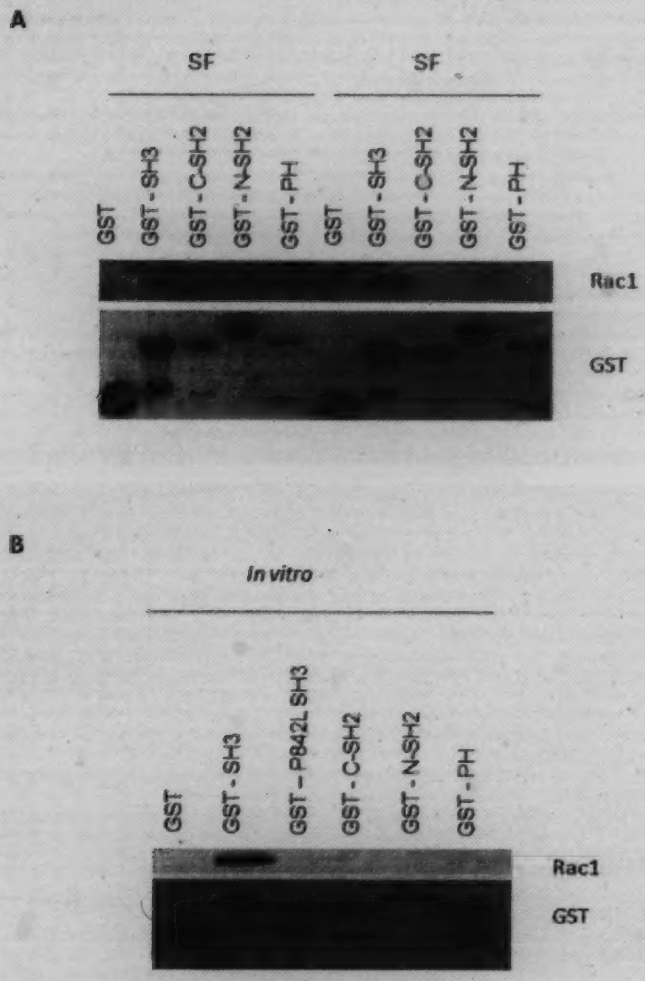


Figure 3.5 SH3 domain of PLC-γ1 mediates the association *in vitro*. (A) The association of Rac1 with various GST fusion PLC-γ1 domains by GST pull-down. COS-7 cells were not treated or treated with EGF (100 ng/ml) for 30 min. The cell lysates were incubated with various GST fusion PLC-γ1 proteins bound to glutathione-agarose beads. Bound proteins were analyzed by SDS-PAGE followed by immunoblotting with anti-Rac1 antibody. Immunoblotting with anti-GST antibody was used as loading controls. (B) The association of Rac1 with various GST fusion PLC-γ1 domains by GST pull-down *in vitro*. Purified Rac1 (50 μM) were incubated with purified GST fusion PLC-γ1 domains *in vitro*. Bound proteins were resolved on SDS-PAGE and visualized by western blots using anti-Rac1 and anti-GST antibodies.

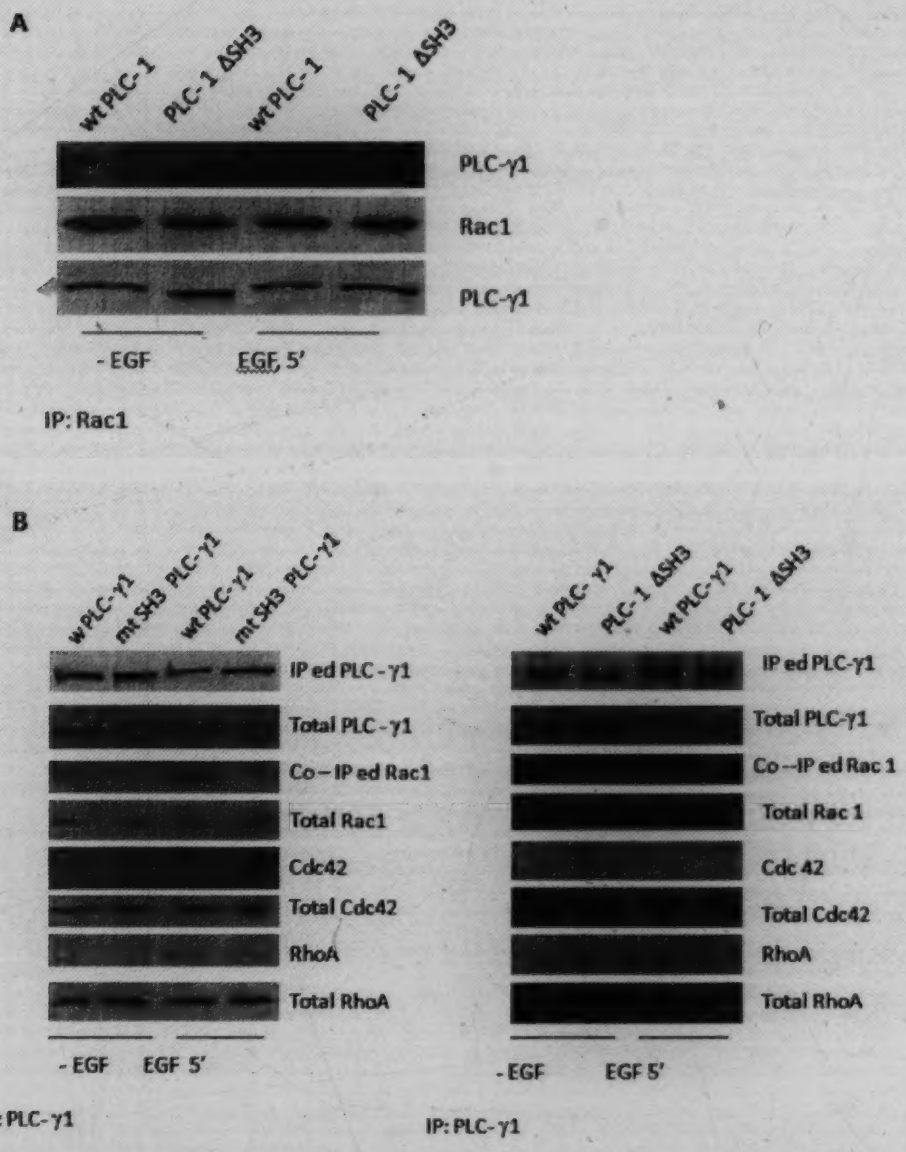


Figure 3.6 SH3 domain of PLC-γ1 is required for association with Rac1 *in vivo*. (A) COS-7 cells were transfected with wild type, or PLC-γ1ΔSH3. The cells were treated with EGF (100 ng/ml) for 5 min. Rac1 were immunoprecipitated with monoclonal anti-Rac1 antibody, and the resulting immunoprecipitates were subjected to immunoblotting with a polyclonal anti-GFP antibody and a polyclonal anti-Rac1 antibody. (B) COS-7 cells were transfected with wt PLC-γ1, mt SH3 PLC-γ1 (P842L), or PLC-γ1ΔSH3 and treated the same as described in (A). YFP-tagged different forms of PLC-γ1 were immunoprecipitated with anti- GFP antibodies and the bound proteins were subjected to SDS-PAGE and stained with different antibodies.

3.4 Rac1 Proline-rich motif ^{106}PNTP^{109} mediates its interaction with PLC-γ1

We then determined which sequences of Rac1 interact with PLC-γ1. We have shown that SH3 domain of PLC-γ1 is responsible for the interaction between PLC-γ1 and Rac1. It has been well established that SH3 domain consensus binding sequences are PXXP. The Rac1 sequences that interact with PLC-γ1 SH3 domain more likely contain PXXP consensus sequence. Analysis of Rac1 sequence reveals the presence of one PXXP motif ^{106}PNTP^{109}. To determine whether this motif indeed mediates the interaction between PLC-γ1 and Rac1, we mutated the two prolines to alanines by site-directed mutagenesis (Fig. 3.7). The GFP-tagged mutant (Rac1 PP/AA) and wild type Rac1 were expressed in COS-7 cells by transient transfection. EGF-stimulated interaction between PLC-γ1 and the mutant was examined by co-IP. We first used monoclonal anti-Rac1 antibody to immunoprecipitate both endogenous Rac1 and transfected GFP-Rac1 Rac1. Immunoblotting of the immunoprecipitates with antibodies to Rac1, GFP and PLC-γ1 showed that PLC-γ1 only co-immunoprecipitated with wild type Rac1 following EGF stimulation (Fig. 3.8A and 3.8B). PLC-γ1 did not co-immunoprecipitated with Rac1PP/AA regardless EGF stimulation (Fig. 3.8A and 3.8B). Parallel experiments were carried out by immunoprecipitating only GFP Rac1 with anti-GFP antibody. Immunoblotting with antibodies to GFP and PLC-γ1 showed similar results, in which PLC-γ1 only co-precipitated with wt GFP-Rac1 (Fig. 3.8C and 3.8D). This suggests that the interaction between PLC-γ1 and Rac1 is mediated by Rac1 ^{106}PNTP^{109}.

We further examined the interaction between PLC-γ1 SH3 domain and Rac1

^{106}PNTP^{109} motif by GST pull-down. COS-7 cells were transfected with wild type Rac1 and mutant Rac1PP/AA, the cell lysates were incubated with GST-fusion PLC-γ1 SH3 domain conjugated to agarose beads. We showed that GST fusion PLC-γ1 SH3 domain specifically pulled down wild type Rac1, but not the Rac1 PP/AA (Fig. 3.8E). Together, our data indicates that PLC-γ1 SH3 domain specifically interacts with Rac1 ^{106}PNTP^{109}.

WT Rac1

MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVN

LGLWDTAGQEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHC

PNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECS

ALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCLLL

PP/AA Rac1

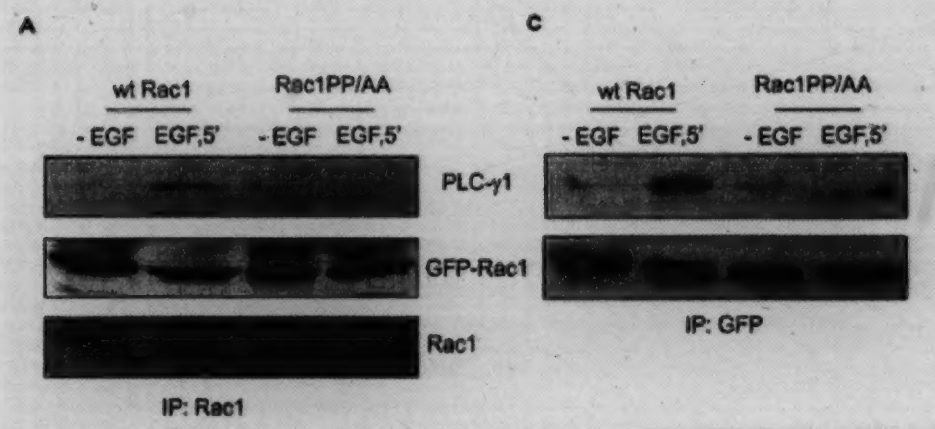
MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVN

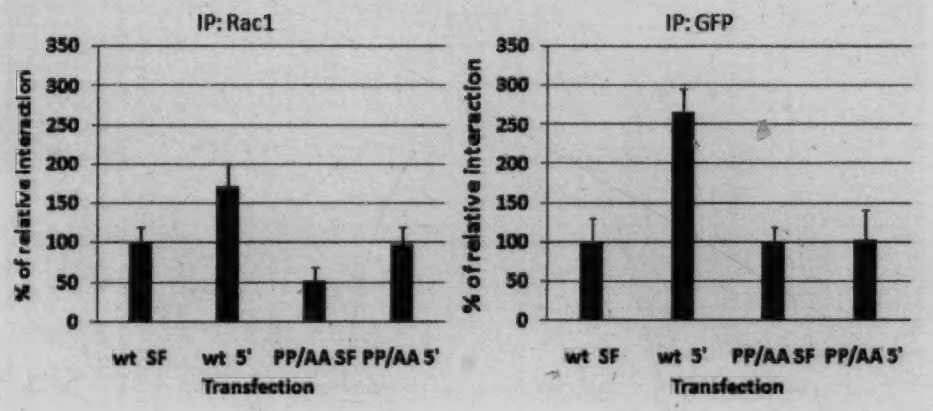
LGLWDTAGQEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHC

ANTAIILVGTKLDLRDDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECS

ALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCLLL

Figure 3.7 The wild type and mutant Rac1 amino acid sequence. In PP/AA mutant Rac1, the two proline residues were mutated to alanines.


A
wt Rac1
Rac1PP/AA
- EGF EGF,5' - EGF EGF,5'
PLC-γ1
GFP-Rac1
Rac1
IP: Rac1
C
wt Rac1
Rac1PP/AA
- EGF EGF,5' - EGF EGF,5'
IP: GFP


B
IP: Rac1
% of relative interaction
350
300
250
200
150
100
50
0
wt SF
wt 5'
PP/AA SF
PP/AA 5'
Transfection
D
IP: GFP
% of relative interaction
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wt SF
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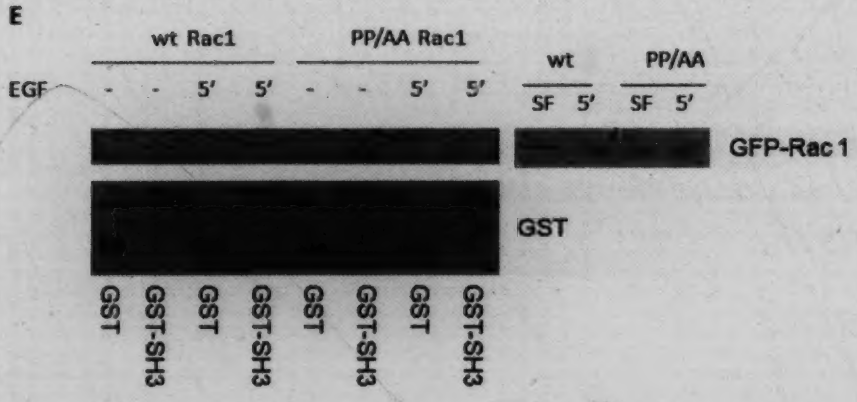
PP/AA SF
PP/AA 5'
Transfection


E
wt Rac1
PP/AA Rac1
wt
PP/AA
EGF - - 5' 5' - - 5' 5'
SF 5' SF 5'
GFP-Rac 1
GST
GST
GST-SH3
GST
GST-SH3
GST
GST-SH3
GST
GST-SH3

3.5 PLC-γ1 activates Rac1 in response to EGF

We next examined whether the EGF-induced interaction between PLC-γ1 and Rac1 results in the activation of Rac1. $P21^{cdc42/rac}$-activated kinase (PAK) is one of the prime downstream effectors of Rac1 and gets activated by active Rac1/Cdc42 but not RhoA in a GTP-dependent manner. PAK contains a PAK Rho binding domain which only binds to activated Rac1/Cdc42, and thus the PAK Rho binding domain in fusion with GST (GST-PAK) has been used to trap Rac1 in their GTP-bound form (Knaus and Bokoch, 1998; Knaus et al., 1998). In our studies, activation of Rac1 was determined by its ability to bind to GST-PAK. We first determined the effects of EGF on Rac1 activation in both BT20 and COS-7 cells. COS-7 and BT20 cells were serum starved for 12 h and stimulated with EGF for various times as indicated. The cell lysates of different conditions were incubated with similar amount of GST-PAK conjugated with agarose beads to pull down activated Rac1. The amount of Rac1 that was pulled down by GST-PAK was revealed by immunobloting with anti-Rac1 antibody. As shown in Figure. 3.9A, Rac1 is activated by EGF and the Rac1 activation reached maximum at 15 min of EGF stimulation for both BT20 and COS-7 cells.

We next examined whether overexpression of PLC-γ1 enhances EGF-induced Rac1 activation. COS-7 cells were transfected with either wild type PLC-γ1 (wt PLC-γ1) or mutant PLC-γ1 lacking SH3 domain (PLC-γ1ΔSH3). Rac1 activation was determined by pull-down with GST-PAK. Immunoblotting with anti-GST and anti-Rac1 antibodies showed that expression of wild type PLC-γ1 enhanced EGF-induced activation of Rac1; however, expression PLC-γ1ΔSH3 did not enhance

EGF-induced activation of Rac1 (Fig. 3.9B). This indicates that PLC-γ1 activates Rac1 *in vivo* in response to EGF and this activation is dependent on its SH3 domain.

We then determined whether Rac1 ^{106}PNTP^{109} is required for EGF-induced activation of Rac1 *in vivo*. Both wild type Rac1 and Rac1 PP/AA were expressed in COS-7 cells by transient transfection. Rac1 activation was determined by pull-down with GST-PAK. Immunoblotting with anti-GST and anti-Rac1 antibodies showed that wild type Rac1 is strongly activated by EGF stimulation, but Rac1 PP/AA was not activated by EGF (Fig. 3.10A). Paralleled experiments were performed using wild type and different mutants of Rac1 (Fig. 3.10B). COS-7 cells were transfected with wt Rac1, Rac1 PP/AA, N17 Rac1 and L61 Rac1. N17 Rac1 contains a threonine to asparagine substitution at residue 17, which results a dominant negative form of Rac1. It is used as a negative control in this research. L61 Rac1 contains a glutamine to leucine substitution at residue 61, which converts wild type Rac1 to a constitutively active form. This mutant is used as a positive control for my research. As expected, N17 Rac1 cannot get activated either with or without EGF stimulation and L61 Rac1 was constitutively active with or without EGF treatment. The wild type Rac1 get activated after EGF stimulation but not Rac1 PP/AA mutant, which is consistent with results shown in Figure 3.10A. Together, our data indicate that EGF stimulates the activation of Rac1 and this stimulation is mostly mediated by the interaction between PLC-γ1 SH3 domain and Rac1 ^{106}PNTP^{109}.

As we showed in Figure 3.10 that Rac1 PP/AA cannot be activated after EGF stimulation. This may indicate that Rac1 PP/AA cannot be activated by PLC-γ1 after

EGF stimulation due to inability of binding to SH3 domain of PLC-γ1, or the PP/AA mutation of Rac1 altered its binding property to GTP resulting in a non-functional Rac1. To examine the binding properties of Rac1 PP/AA with GTP, we transfected COS-7 cells with Rac1 PP/AA and wt Rac1, and the cell lysate containing Rac1 PP/AA with 10 mM EDTA followed by 100 μM GTP-γS for 15min, and then 60 mM $MgCl_2$ were added to terminate the reaction. Following that the cell lysates were subjected to a GST-PAK pulldown assay. Meanwhile, N17 Rac1 and L61 Rac1 were trasfected into COS-7 cells and cell lysates containing each were subjected to GST-PAK pulldown as negative and positive controls, respectively. Figure 3.11 showed that GST-PAK pulled down similar amount of wild type Rac1 and Rac1 PP/AA which indicating that both wild type and Rac1 PP/AA have same ability to bind to GTP-γS. As expected GST-PAK did not pulldown N17 Rac1, but pulled down significant amount of L61 Rac1. All together, the data suggested that Rac1 PP/AA can still bind to GTP with similar ability as wild type Rac1. Therefore, inability of Rac1 PP/AA from getting activated under EGF stimulation is due to the inability of binding to PLC-γ1.

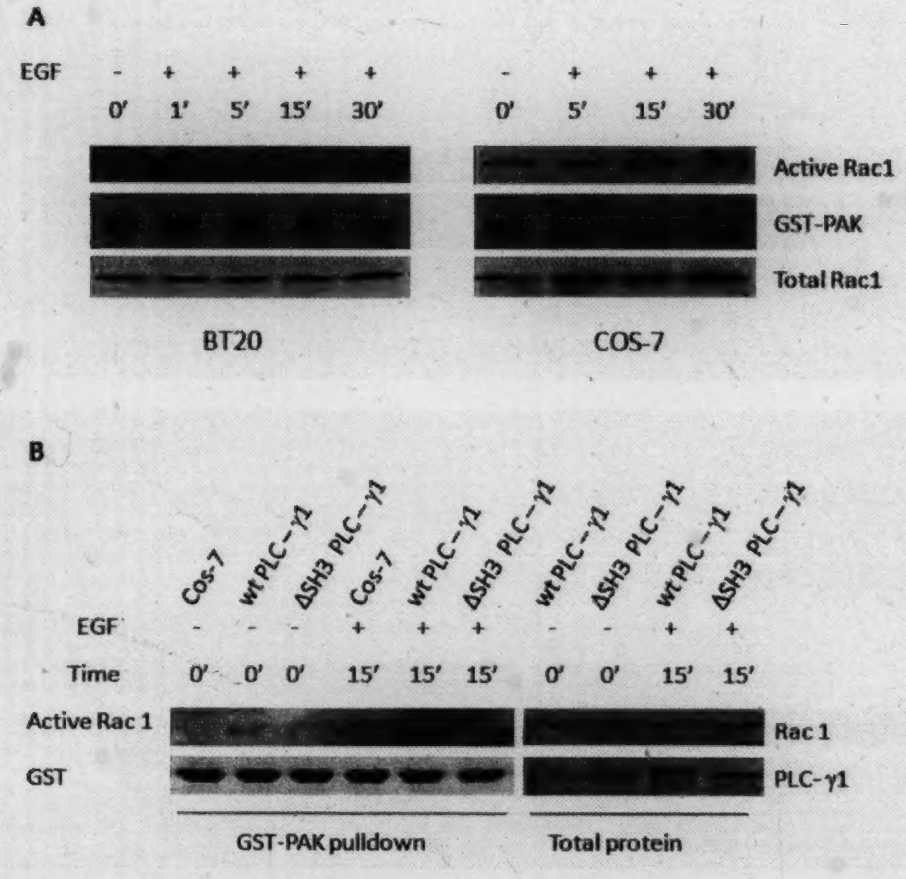


Figure 3.9 The effect of PLC-γ1 SH3 domain on EGF-induced activation of Rac1. The activation of Rac1 was determined by pull down with GST-PAK. Rac1 binding domain as described in Materials and Methods. Briefly, the cell lysates were incubated with GST-PAK bound to glutathione-agarose beads. Bound proteins were analyzed by immunoblotting with anti-Rac1 antibody. Immunoblotting with anti-GST antibody was used as loading control. (A) EGF-induced activation of Rac1 in BT20 and COS-7 cells. BT20 and COS-7 cells were stimulated with EGF for indicated time and the activation of Rac1 was determined as above. (B) The effects of PLC-γ1 SH3 domain on EGF-induced activation of Rac1. COS-7 cells were transfected with YFP-tagged wild type PLC-γ1 or mutant PLC-γ1ΔSH3. The cells were either not treated or treated with EGF (100 ng/ml) for 30 min. The cells incubated with GTPγS were used to determine the maximum activation of Rac1. The Rac1 activation was determined as above.

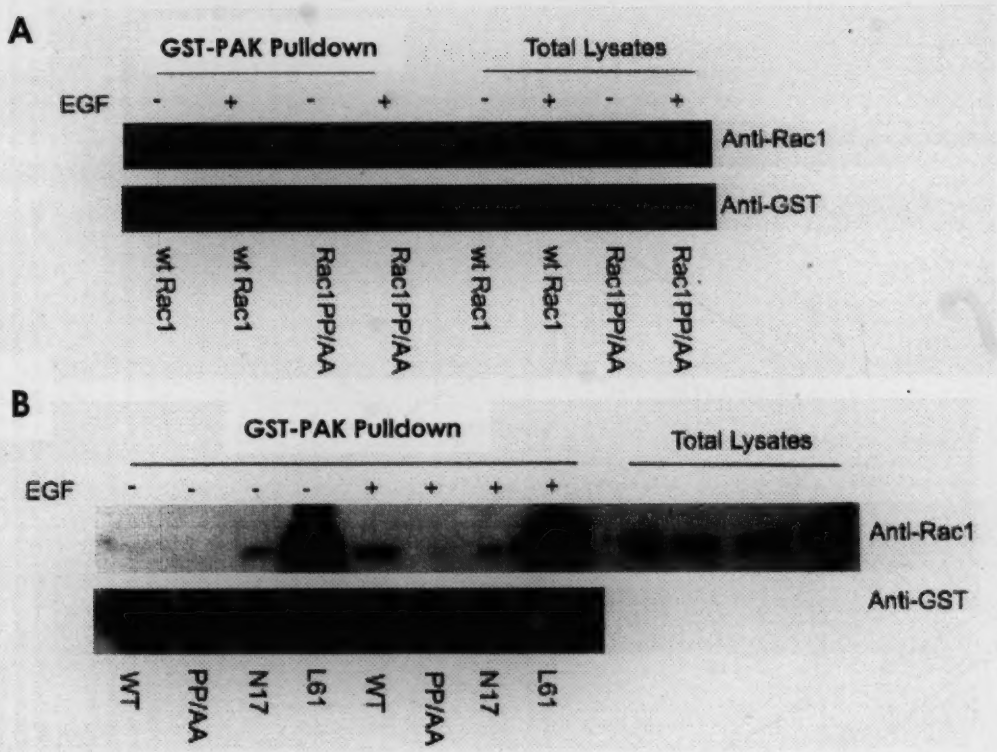


Figure 3.10 The effect of PP/AA mutantion on EGF-induced activation of Rac1. The activation of Rac1 was determined by pull down with GST-PAK Rac1 binding domain as described in Materials and Methods. Briefly, the cell lysates were incubated with GST-PAK bound to glutathione-agarose beads. Bound proteins were analyzed by immunoblotting with anti-Rac1 antibody. Immunoblotting with anti-GST antibody was used as loading control. (A) The effects of PLC-γ1 SH3 domain on EGF-induced activation of Rac1. COS-7 cells were transfected with GFP-tagged wild type Rac1 or mutant Rac1 PP/AA. The cells were either not treated or treated with EGF (100 ng/ml) for 15 min. The Rac1 activation was determined as above. (B) The effect of mutations on Rac1 activation. The COS-7 cells were transfected with wt Rac1, Rac1 PP/AA, dominant negative N17 Rac1 and constitutive active L61 Rac1. The cells were either treated or not treated with EGF (100 ng/ml) for 15 min. Active Rac1 were pulled down by GST-PAK.

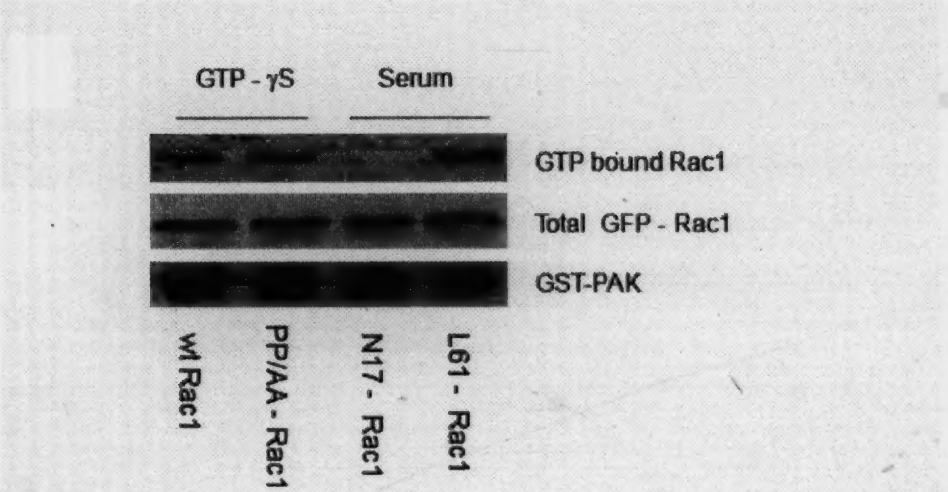


Figure 3.11 PP/AA Rac1 binds to GTP-γS with similar affinity compared to wt Rac1. COS-7 cells were transfected with different forms of GFP-Rac1 and then lysed. Cell lysates containing L61 Rac1 and N17 Rac1 were subjected directly to a GST-PAK pulldown, while cell lysates containing wt Rac1 and PP/AA Rac1 were treated with EDTA (10 mM) for 5 min then GTP-γS (100 μM) for 15 min followed by adding $MgCl_2$ to final of 60 mM, then the lysate was subjected to GST-PAK pulldown. Active GTP-Rac1 is visualized by immunoblotting with anti-GFP antibodies.

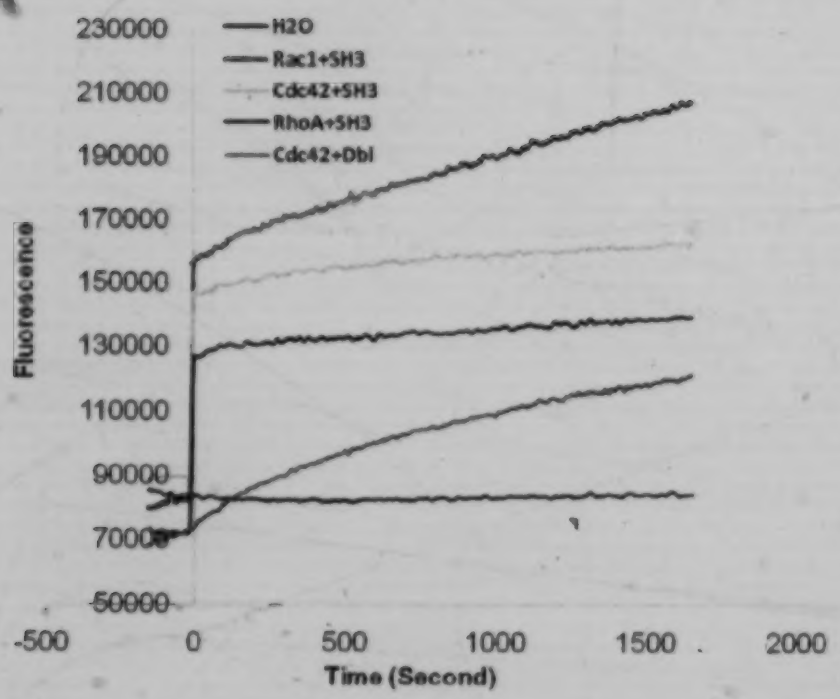
3.6 PLC-γ1 SH3 domain is a GEF for Rac1 *in vitro*

Our above data indicated that PLC-γ1 mediates EGF-induced activation of Rac1 by a direct interaction through its SH3 domain and Rac1 ^{106}PNTP^{109} motif. It has been shown recently that the PLC-γ1 SH3 domain acts as a guanine nucleotide exchange factor (GEF) for PIKE and dynamin-1 (Ye et al., 2002; Choi et al., 2004). Thus, it is possible that the function of PLC-γ1 in EGF-induced Rac1 activation is due to a direct GEF activity. To determine whether PLC-γ1 SH3 domain is a GEF for Rac1, an *in vitro* nucleotides exchange assay was performed by using RhoGEF exchange assay kit (Cytoskeleton, Inc., USA). PLC-γ1 SH3 domain was purified from GST-PLC-γ1 SH3 domain by cutting off the GST with Factor Xa and all other proteins came with the kit. This fluorescence based assay uses a fluorescent analog called mant (N-methylanthraniloyl) fluorophores. It takes advantage of the spectroscopic difference between bound and unbound mant-fluorophores to guanine nucleotides and thus is able to monitor the states of small GTPases (Cheng et al., 2002; Rossman et al., 2002). Excitation of mant flurophore at 360 nm (+/- 10 nm) gives a fluorescence emission at 440 nm (+/- 20 nm). Once bound to GTPases, the fluorophore emission intensity will increase approximately 2 fold. Thus in this experiment, the enhancement of fluorescent intensity in the presence of different Rho GTPases (Rac 1, Cdc42 and RhoA) and SH3 domain will reflect the respective GEF activities of SH3 domains.

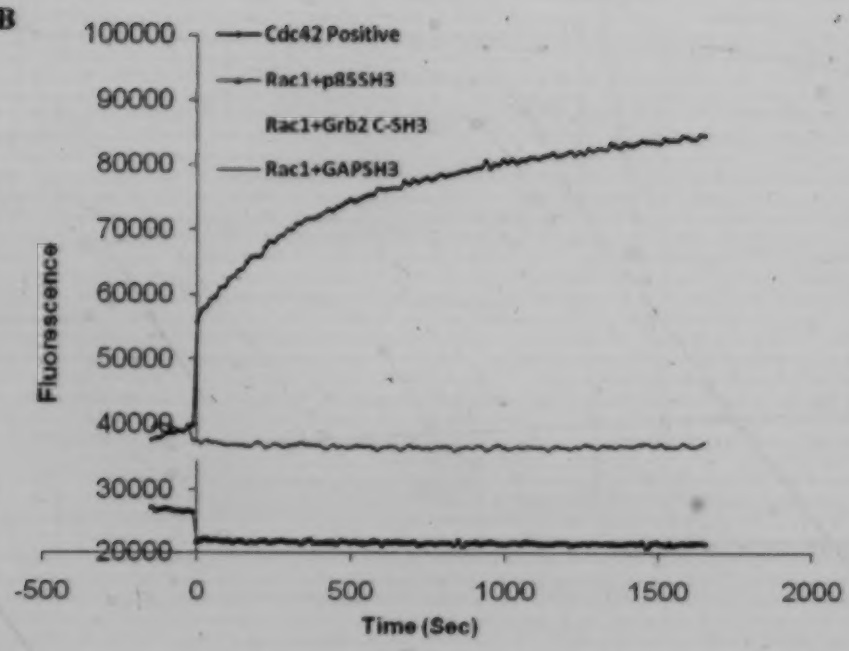
As shown in Figure. 3.11A and 3.11C, the fluorescence intensity increased dramatically when purified SH3 domains were added to Rac1 but not for Cdc42 and RhoA. Dbl proteins were used as positive controls for different Rho GTPases and

water was used as a negative control. Together, the data suggested that PLC-γ1 SH3 domain showed strong GEF activity towards Rac1. The exchange activity of PLC-γ1 SH3 domain on Rac1 is approximately 60% of the GEF activity of Dbl on Cdc42. However, the exchange activity of PLC-γ1 SH3 domain on RhoA and Cdc42 is much lower than that of Rac1. The exchange activity of PLC-γ1 SH3 domain on RhoA is almost 6-fold lower than that of Rac1 and the exchange activity of PLC-γ1 SH3 domain on Cdc42 is only half of that of Rac1. These data indicate that PLC-γ1 SH3 domain is a specific GEF for Rac1. SH3 domain is a common domain present in various signaling proteins. Although its binding consensus sequence is well defined, different SH3 domains may have different function. Therefore, we examined if only the SH3 domain from PLC-γ1 is a specific GEF for Rac1. To do that, we purified several SH3 domains from other proteins including GAP SH3 domain, p85 SH3 domain, and Grb2 C-SH3 domain and subjected them to the same *in vitro* GEF activity assay described above. As shown in Figure 3.11B and 3.11C, all these SH3 domains did not have GEF activity on Rac1. The data suggested that only PLC-γ1 SH3 domain has GEF activity on Rac1.

A


H2O
Rac1+SH3
Cdc42+SH3
RhoA+SH3
Cdc42+Dbl
230000
210000
190000
170000
150000
130000
110000
90000
70000
50000
Fluorescence
-500
0
500
1000
1500
2000
Time (Second)

B


Cdc42 Positive
Rac1+p85SH3
Rac1+Grb2 C-SH3
Rac1+GAPSH3
100000
90000
80000
70000
60000
50000
40000
30000
20000
Fluorescence
-500
0
500
1000
1500
2000
Time (Sec)

3.7 PLC-γ1's lipase activity is not required for its GEF activity on Rac1 and PLC-γ1 binds to inactive Rac1 with higher affinity

PLC-γ1 is well characterized as a lipase on PIP2, so another concern of this project is whether the lipase activity of PLC-γ1 is required for Rac1 activation. We transfected wild type PLC-γ1 or ΔSH3 PLC-γ1 to COS-7 cells and then treated the cells with U73122, a known lipase inhibitor specific to PLC-γ1, for half an hour followed by EGF treatment. Cells with or without U73122 treatments were lysed and subjected to GST-PAK pulldown assay described before. Figure 3.12A showed that wild type PLC-γ1 over-expression increased the amount of active Rac1 compared to non-transfected cells and ΔSH3 PLC-γ1 decreased the amount of active Rac1 due to its negative dominant effect. Furthermore, with or without U73122 treatment, GST-PAK pulls down similar amount of active Rac1 for each transfection with different treatment. Same experiments were repeated 5 times and results were quantified in Figure 3.12A. These data suggested that PLC-γ1's lipase activity is not required for Rac1 activation.

Finally we examined the binding affinity of PLC-γ1 to different mutants of Rac1 as GEF binds to inactive GTPase with higher affinity. We transfected cells with wild type Rac1, N17 Rac1 and L61 Rac1 and the cells containing transfections were lysed and subjected to GST-SH3 pulldown assay. As shown in Figure 3.12B, GST-SH3 binds N17 Rac1, a constitutively inactive form, with higher affinity compared with L61 Rac1, a constitutively active form of Rac1. Together, our data strongly suggests that PLC-γ1 SH3 domain is a Rac1 GEF.

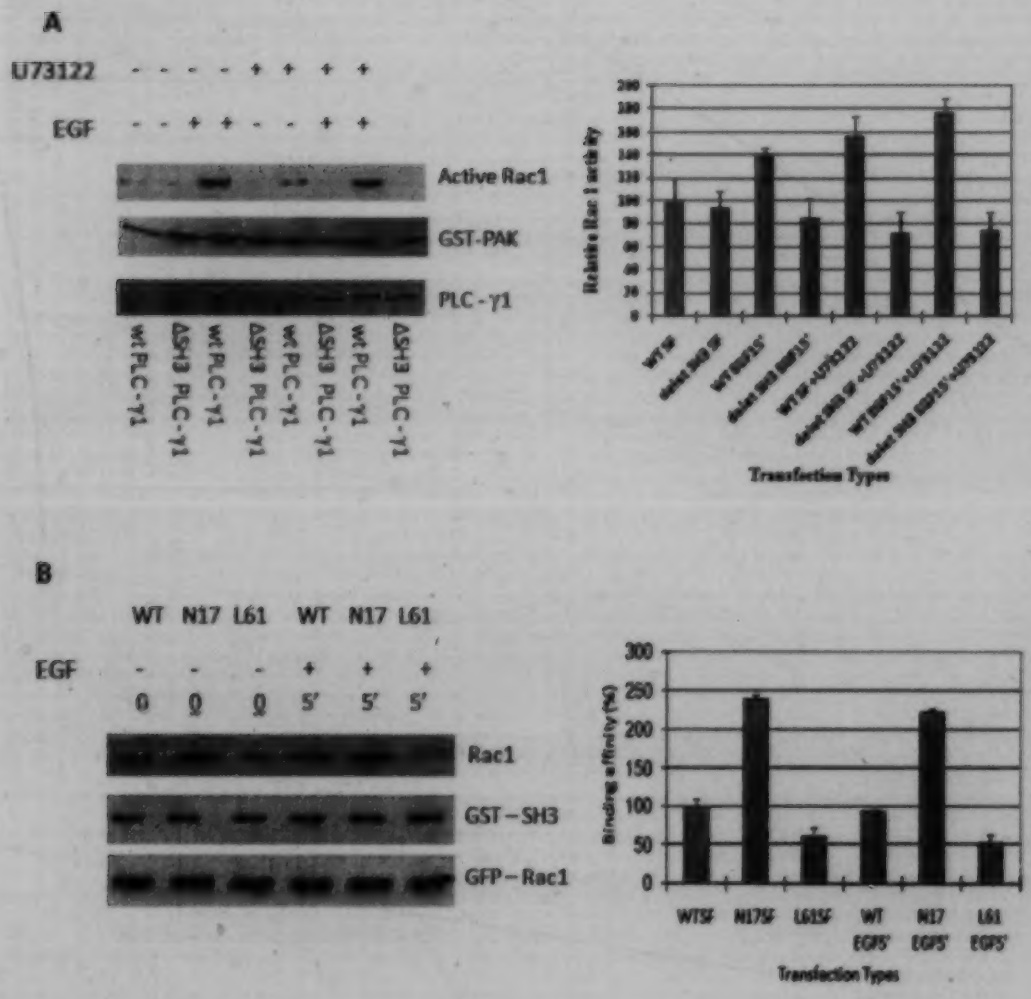


Figure 3.13 PLC-γ1 SH3 domain function as a GEF for Rac1 *in vivo*. (A) The GEF activity of PLC-γ1 SH3 domain on Rac1 was determined by GST-PAK pulldown assay as described in Materials and Methods. COS-7 cells with different transfections were either treated with U73122 for 30 min followed by EGF for 15 min or EGF alone for 15 min. Active Rac1 was pulled down by GST-PAK in each conditions. (B) The PLC-γ1's SH3 domain binds to inactive Rac1 N17 with higher affinity.

3.8 Regulation of EGF-induced cell motility by PLC-γ1 SH3 domain and Rac1 interaction

It was shown that both PLC-γ1 and Rho GTPases controls cell motility by regulating the reorganization of cytoskeleton in response to EGF (Sahai and Marshall, 2002; Etienne-Manneville and Hall, 2002). As shown above, PLC-γ1 is a GEF for Rac1 both *in vitro* and *in vivo*, it would be interesting to examine whether PLC-γ1 could regulate cell motility by its interaction with Rac1. To test this possibility, we first determined whether PLC-γ1 SH3 domain is important in EGF-induced cell motility. We transfected COS-7 cells with either wild type PLC-γ1 or PLC-γ1ΔSH3 tagged with YFP. Then EGF-induced cell migration was examined by wound healing assays. The migration of 50 transfected cells and 50 non-transfected cells were measured and statistically analyzed. The data was shown in Figure 3.14 that over-expression of wild type PLC-γ1 significantly enhanced EGF-induced cell motility by 50%. However, deletion of PLC-γ1 SH3 domain not only abolished this enhancement, but also significantly inhibited EGF-induced cell motility (Fig. 3.14A and 3.14B). These results suggest that PLC-γ1 SH3 domain accounts for a significant part of cell migration induced by PLC-γ1. We then examined whether overexpression of wild type Rac1 in COS-7 cells increase the cell motility and whether the effects are dependent on its interaction with PLC-γ1. COS-7 cells were transfected with either wild type Rac1 or mutant Rac1 PP/AA tagged with GFP. The wound healing assay as described above showed that overexpression of wild type Rac1 increased cell motility by 30% (Fig. 3.15A and 3.15B). However, transfection of COS-7 cells with mutant Rac1 PP/AA

that does not bind to PLC-γ1 significantly inhibited EGF-induced cell motility (Fig. 3.15A and 3.15B). This suggests that the enhancement of Rac1 on EGF-induced cell motility is dependent on its interaction with PLC-γ1. Together, these results suggest that PLC-γ1-Rac1 interaction plays an important role in EGF-induced cell motility.

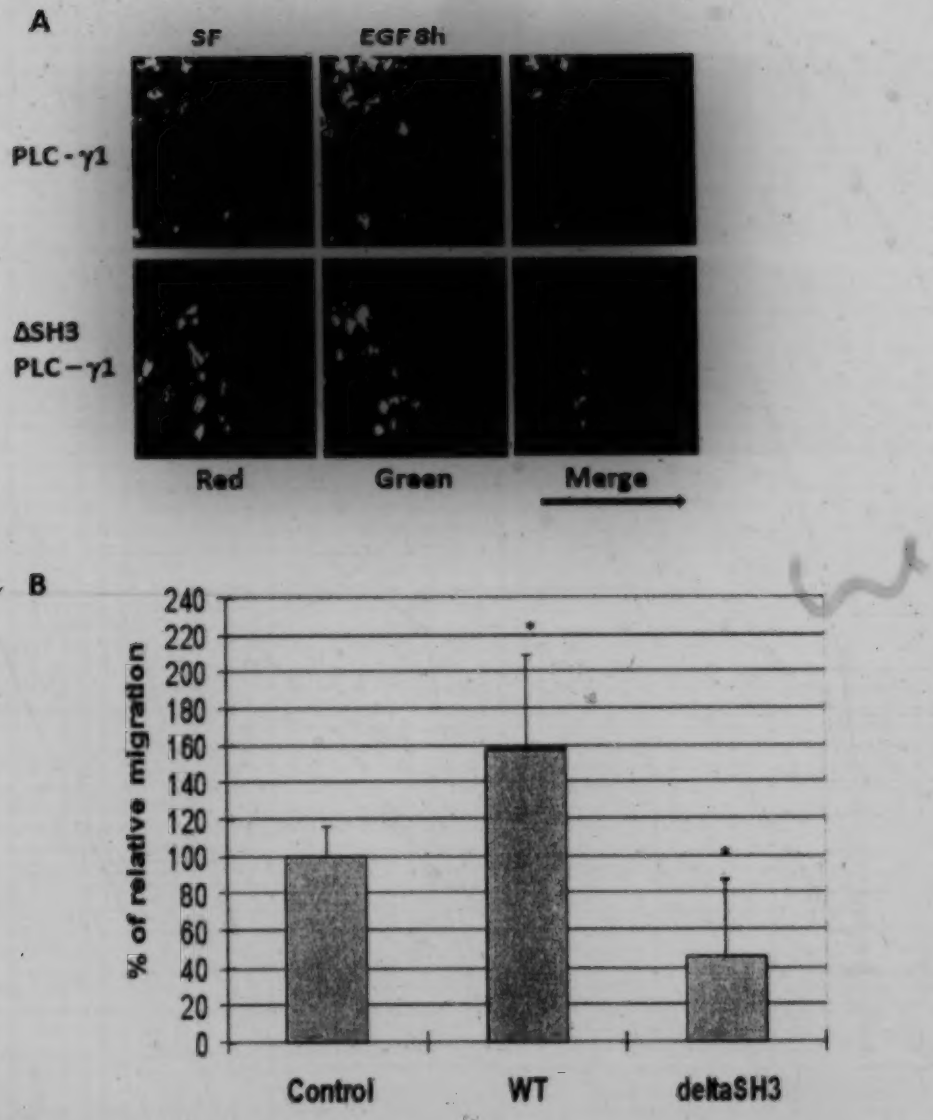


Figure 3.14 Effects of PLC-γ1 SH3 domain on EGF-induced cell motility. (A) COS-7 cells were transfected with YFP-tagged wild type PLC-γ1 or mutant PLC-γ1ΔSH3. Confluent monolayer serum starved cells were treated with EGF (100 ng/ml) as indicated. The cell motility was examined by wound healing assay as described in Materials and Methods. In the merged image, red showed the cells at 0 h and the green showed the cells at 8 h. All of cells migrating from the left to right as indicated. (B) Quantitation of the data from (A). The data was quantitated as described in Materials and Methods with n=50. Statistical significance is indicated as * for $p<0.0001$

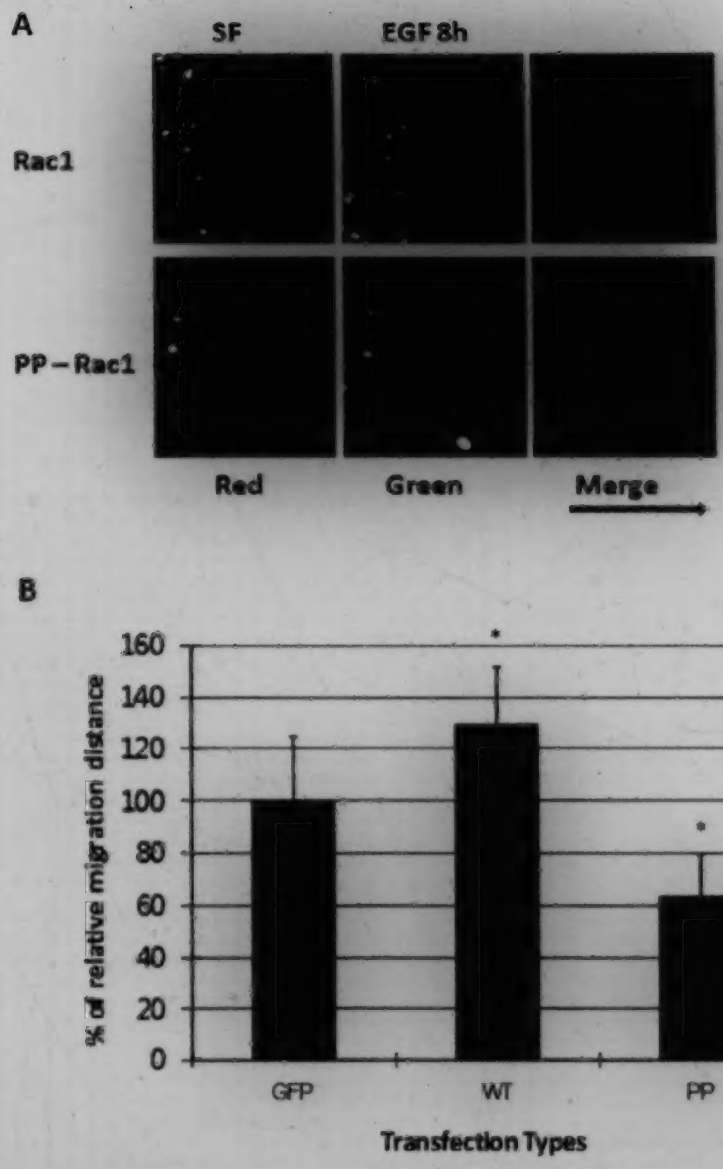


Figure 3.15 Mutation of Rac1 ^{106}PNTP^{109} and the effect on EGF-induced cell motility. (A) COS-7 cells were transfected with GFP-tagged wild type Rac1 or mutant Rac1PP/AA. Confluent monolayer serum starved cells were treated with EGF (100 ng/ml) as indicated. The cell motility was examined by wound healing assay as described in Materials and Methods. In the merged image, red showed the cells at 0 h and the green showed the cells at 8 h. All of cells migrating from the left to right as indicated. (B) Quantitation of the data from (A). The data was quantitated as described in Materials and Methods with n=50. Statistical significance is indicated as * for $p<0.0001$

Chapter 4

Discussion

It is well established that EGF stimulates cell motility by activated EGFR and downstream signaling pathways. PLC-γ1 pathways, and Rac1 pathway are the most important and well characterized pathways regulating EGFR-mediated cell motility (Wells et al., 2002; Ridley and Hall, 1992a; Ridley and Hall, 1992b; Ridley et al., 1992e; Ridley et al., 1992d; Malliri and Collard, 2003; Banyard et al., 2000; Piccolo et al., 2002). While it is known that PLC-γ1 and Rac1 coordinate EGF-induced cell movement, no direct connections between two proteins have ever been reported. In this study we for the first time demonstrated a direct connection between these two proteins both *in vivo* and *in vitro* and showed the importance of this interaction in EGF-induced cell movement.

We first determined whether PLC-γ1 and Rac1 directly interact in response to EGF. It is not surprising to see the colocolization between PLC-γ1 and Rac1 at the plasma membrane (Fig. 3.1) for it has been shown previously that functional Rac1 is mainly localized to the plasma membrane and PLC-γ1 translocates to the plasma membrane in response to EGF (Wang and Wang, 2003). Knock-down of endogenous PLC-γ1 did not alter Rac1 localization with or without EGF stimulation, which indicates the localization of PLC-γ1 and Rac1 is in a PLC-γ1 translocation dependent manner and Rac1 membrane localization is not mediated by PLC-γ1 (Fig. 3.2). Furthermore, a mutant PLC-γ1 lacking the SH3 domain can also translocate to plasma membrane and co-localize with Rac1 after EGF stimulation (Fig. 3.3). This suggests that the SH3 domain deletion does not affect PLC-γ1 translocation or co-localization with Rac1 after EGF stimulation. Co-immunoprecipitation experiments showed that

PLC-γ1 formed complex with Rac1 in EGF-dependent manner and the complex formation reached maximum at 5 and 15 min and retained until 30 min of EGF stimulation (Fig. 3.4). Together, these data suggest that PLC-γ1 and Rac1 interact at the plasma membrane to regulate cell movements in an EGF dependent manner.

We next examined which domain of PLC-γ1 is responsible for binding to Rac1. We showed by both *in vitro* and *in vivo* protein association experiments that PLC-γ1 SH3 domain is essential for the binding (Fig. 3.5). We first conducted *in vitro* GST-pull down experiments and showed that only GST fusion PLC-γ1 SH3 domain can pull down Rac1 in a EGF independent manner, but not any other individual domain (Fig. 3.5A). This interaction between Rac1 and SH3 domain was further confirmed by GST-Pull down using a non-functional SH3 domain containing P842L mutation, which disrupted for association (Fig 3.5B). We then showed that *in vivo* two mutants of PLC-γ1, PLC-γ1 lacking SH3 domain (PLC-γ1 ΔSH3) and PLC-γ1 containing P842L SH3 domain, both failed to co-immunoprecipitate with Rac1 with or without EGF stimulation, however, the full-length wild type PLC-γ1 co-immunoprecipitated with Rac1 but not RhoA or Cdc42 after EGF stimulation (Fig. 3.6). It is interesting to note that PLC-γ1 SH3 domain interaction with Rac1 is dependent on EGF stimulation *in vivo* but not *in vitro*. While the *in vivo* and *in vitro* data seem at odds, similar observations have been reported previously. For example, the interaction between PLC-γ1 SH3 domain and PLD_2 (Jang et al., 2003), PIKE (Ye et al., 2002), dynamin (Choi et al., 2004), and Emt (Perez-Villar and Kanner, 1999) are all dependent on EGF stimulation. In fact, we recently observed similar interaction between PLC-γ1 and Akt

(Wang et al., 2006). In that study our data suggest a model that *in vivo* the full length PLC-γ1 adopts a conformation that restricts the interaction of its SH3 domain with Akt under non-EGF stimulated condition. Phosphorylation of PLC-γ1 on Y771 and/or Y783 by EGF stimulation releases the restriction on SH3 domain. However, *in vitro*, the GST-fusion PLC-γ1 SH3 domain is not subject to this restriction (Wang et al., 2006). We believe that our finding here regarding PLC-γ1 and Rac1 interaction could also be explained by this model. Therefore, the interaction between PLC-γ1 and Rac1 requires EGFR engagement and PLC-γ1 activation under physiological conditions.

Followed the identification of PLC-γ1 SH3 domain as responsible for interacting with Rac1, we next investigated which sequence of Rac1 is responsible for the interaction. Given that the SH3 domain minimum binding sequences were well characterised to PXXP, we examined Rac1 amino acid sequence and identified ^{106}PNTP^{109} motif. We generated a mutant Rac1 with both prolines were replaced by alanines (Rac1 PP/AA) (Fig. 3.7). We also generated a dominant negative mutant N17 and constitutively active mutant L61 as controls. Protein binding assays including GST-pulldown and IP were performed to test the binding ability of Rac1 PP/AA mutant to SH3 domains (Fig. 3.8). Our data suggested *in vivo* mutation of the two prolines to alanines on Rac 1 significantly reduced the interaction between wild type PLC-γ1 and Rac1 after EGF stimulation (Fig. 3.8A and 3.8B). This finding was further conformed by *in vitro* binding assay, in which GST-SH3 protein can only associate with wild type Rac1 in an EGF independent manner (Fig. 3.8E). These findings were consistent with our data shown in Figure 3.6 and can also be explained by the model

established previously (Wang et al., 2006).

The most significant finding of this research is that PLC-γ1 SH3 domain is a GEF for Rac1. We first demonstrated the activation of Rac1 by EGF stimulation in two different cell lines – COS-7 and BT-20, which expresses modulate and high level of EGFR respectively. In both COS-7 and BT-20 cells, Rac1 was activated by EGF stimulation (Fig. 3.9A). We then showed that *in vivo*, over-expression of PLCγ1 enhances EGF-induced activation of Rac1 and that this enhancement is dependent on PLC-γ1 SH3 domain interaction with proline motif of Rac1 (Fig. 3.9B), because either deletion of SH3 domain or mutation of the proline rich motif of Rac1 dramatically reduced the activation of Rac1 following EGF stimulation (Fig. 3.9 and 3.10). However, there are two possibilities why mutant PP/AA Rac1 fails to get activated after EGF. One possibility is the mutant form of Rac1 alters its binding affinity to GTP, which results in a non-activated form, another possibility is the mutant PP/AA Rac1 can still bind to GTP with same ability but the inactivation by EGF is due to inability of binding to PLC-γ1. We therefore tested the two possibilities by a GST-PAK pulldown assay, and with GTP-γS pre-loading, wild type Rac1 and PP/AA Rac1 bind to GTP-γS with similar affinity (Fig. 3.11). Thus, we concluded that the PP/AA mutation does not affect Rac1 GTP binding but affect Rac1 activation by blocking the interaction with PLC-γ1 SH3 domain.

Moreover, we showed by *in vitro* GEF assay that PLC-γ1 SH3 domain is a moderate and specific GEF for Rac1 (Fig. 3.12). So far, very few GEFs for Rho GTPases have been identified. It is know that Tiam1 is a GEF for RhoA, Rac1 and

Cdc42 (Habets et al., 1994; Hoffman and Cerione, 2002). The best characterized GEFs for Rac1 are Vav proteins. Vav1 is restricted in its distribution to hematopoietic cells and has been shown to act on Rac1, Cdc42, and RhoA *in vitro* (Bustelo, 1996; Bustelo, 2000). Vav2 is ubiquitously distributed and has shown to activate Rac1, Cdc42, and RhoA downstream from growth factor receptor (Liu and Burridge, 2000). Here we showed that PLC-γ1 also activates Rac1 downstream of EGFR, and the exchange activity of PLC-γ1 SH3 domain on Rac1 is very similar to that of Vav2 demonstrated by Liu and Burridge (Liu and Burridge, 2000). However, Vav2 is a GEF for the three members of Rho family: RhoA, Rac1 and Cdc42. Our data suggested that PLC-γ1 is a much better GEF for Rac1 than for Cdc42 and RhoA (Fig. 3.12). While *in vitro* GEF assay showed that PLC-γ1 SH3 domain possesses some GEF activity on Cdc42, no detectable interaction between PLC-γ1 and Cdc42 were observed by coimmunoprecipitation assay *in vivo*. This indicates that PLC-γ1 is not a GEF for Cdc42 *in vivo*. It is not surprising that SH3 domain can cross interact with Cdc42 *in vitro* due to the 70% of sequence homology between Cdc42 and Rac1. Finally, by acting as a specific GEF for Rac1, PLC-γ1 provides a means for cells to selectively activate Rac1 without activating the other Rho proteins. Previously, it was reported that PLC-γ1 phosphalipase activity is important in EGF-induced Rac1 activation (Nogami et al., 2003). However, we showed here that inhibition of PLC-γ1 lipase activity by treatment with U73122 did not significantly block EGF-induced Rac1 activation. This suggests that PLC-γ1 mostly activates Rac1 by acting directly as a GEF.

While PLC-γ1 has been show to be a GEF for PIKE and dynamin1 (Choi et al., 2004; Ye et al., 2002), our finding is the first report to show that the PLC-γ1 SH3 domain is also a GEF for small GTPases Rac1. To date, the PLC-γ1 SH3 domain is the only SH3 domain known to possess GEF activity. The GEF activity of PLC-γ1 SH3 domain seems quite unique as many other SH3 domains including Grb2 SH3, Fyn SH3 show no GEF activity towards PIKE and dynamin 1 (Choi et al., 2004; Ye et al., 2002). Here in this study, we also showed GAP SH3, Grb2 C-SH3 and p85 SH3 revealed no GEF activity on Rac1 *in vitro* (Fig. 3.12). However, litter is known how PLC-γ1 SH3 domain could function as a specific GEF.

The nucleotide exchange reaction involves dissociation of GDP and the Mg^{2+} ion and the formation of a nucleotide free intermediate (Cherfils and Chardin, 1999). We also examined the binding activity of PLC-γ1 to N17 Rac1 or L61 Rac1 as GEF proteins have higher affinity to GDP bound inactive GTPases (Cherfils and Chardin, 1999). Results in this study also confirmed this GEF property of PLC-γ1 as Figure 3.13 showed that PLC-γ1 binds to N17 Rac1 with much higher affinity than with L61 Rac1.

We finally showed that disruption of PLC-γ1 and Rac1 interaction significantly blocked EGF-induced cell migration (Fig. 3.14 and 3.15). It is well documented that both PLC-γ1 and Rac1 mediate EGF-induced cell motility (Piccolo et al., 2002; Wells et al., 1998; Maddala et al., 2003). PLC-γ1 phospholipase activity has been suggested to regulate Rac1 activity (Nogami et al., 2003) and cell motility (Wells et al., 2002) although this study showed it is not mandatory for the direct activation of Rac1. PLC-γ1 SH3 domain interacts with Akt, which regulate cell motility (Wang et al.,

2006). Vav proteins have been shown to regulate EGF-induced activation of Rac1 to control cell motility (Liu and Burridge, 2000; Bustelo, 1996). Here we provided evidence to show that the direct interaction between PLC-γ1 and Rac1 is another important mechanism to regulate EGF-induced cell migration. Our data not only provides a direct functional link between PLC-γ1 and Rac1 in EGF-induced cell migration, but also revealed the mechanism of this function link. PLC-γ1 acts as a GEF for Rac1 in response to EGF to activate Rac1. Our novel findings together with previous findings demonstrate that cell possesses multiple ways to regulate motility through the activation of PLC-γ1 and Rac1. The multiple mechanisms may ensure the proper control of cell on EGF-induced motility in the event that one mechanism failed.

In summary, in this study, we first successfully demonstrated co-localization of PLC-γ1 and Rac1 in COS-7 cells in an EGF-dependent manner. The EGF-dependent manner is mainly due to the PLC-γ1 translocation as Rac1 always localized on plasma membrane, and SH3 domain is not required for PLC-γ1's translocation. We then identified a direct interaction between PLC-γ1 and Rac1 and also showed that SH3 domain of PLC-γ1 and proline rich motif of Rac1 are regions essential for this interaction both *in vitro* and *in vivo*. We also revealed that PLC-γ1 is a GEF for Rac1 GTPase both *in vitro* and *in vivo*. Furthermore, we showed this GEF activity is mediated by its SH3 domain interaction with Rac1 proline motif in an EGF-dependent but PLC-γ1 lipase activity independent manner. Finally, we demonstrated that the interactions between PLC-γ1 and Rac1 are important for EGF-stimulated cell migration.

Based on all of our data, we established a model to show how PLC-γ1 regulates cell motility after EGF stimulation. In our final model, PLC-γ1 translocates to plasma membrane and gets phosphorylated after EGFR engagement, and this phosphorylation releases the restriction on SH3 domains resulting in its contacting and activating plasma membrane located Rac1. Rac1 then activates the downstream effectors to produce membrane protrusions at the leading edge of migrating cells.

Although my results demonstrated that phospholipase C-γ1 is a GEF for Rac1 to regulate EGF-induced cell motility, further studies are needed to reveal the molecular mechanisms by which SH3 domain of PLC-γ1 acts as specific GEF for Rac1. Previous study has shown that the GEF activity of PLC-γ1 SH3 domain seem quite unique as many other SH3 domains including Grb2 SH3, Fyn SH3 showed no GEF activity towards PIKE and dynamin 1 (Choi et al., 2004; Ye et al., 2002). In this study, we also tested other proteins containing SH3 domains and showed SH3 domains from several other proteins fails to activate Rac1 *in vitro*, thus we demonstrated SH3 domain from PLC-γ1 is specific GEF for Rac1 *in vitro*. We also showed that SH3 domain of PLC-γ1 is a specific GEF for Rac1, but not RhoA and Cdc42. It would be very interesting to find out what determine this specificity in the future research. Since the minimum sequence of SH3 binding sequence is known to be PXXP, more information about other amino acids in SH3 domain that around the PXXP binding groove should be examined. Alignment of the PLC-γ1 SH3 domain primary sequence with other SH3 domains should be considered for identify possible important amino acids responsible for specific binding to Rac1. Similarly, amino acids flanking the PXXP sequence in

Rac1 should also be examined to identify possible important sequences that responsible for the specificity. Once possible targets amino acids were identified, different point mutations will be constructed and the interaction between the SH3 domain and Rac1 will be examined by GST-pulldown.

Like all members of the small GTPases superfamily, the regulatory cycle of Rac1 is exerted by three distinct families of proteins, GEF, GAP and GDI as described in this study earlier. Recent findings suggest that additional regulatory mechanisms such as phosphorylation might further contribute to the tight regulation of Rho GTPases (Loirand et al., 2006). RhoA was the first Rho protein shown to be phosphorylated. The cAMP-dependent protein kinase (PKA) and the PKG phosphorylate RhoA on serine at position 188 *in vitro* and *in vivo* (Lang et al., 1996; Sauzeau et al., 2000). Phosphorylation of RhoA on S188 does not modify its interaction with GEFs and GAPs. Therefore, it does not affect GDP/GTP exchange and activation (Ellerbroek et al., 2003). However, S188 phosphorylation significantly increases its interaction with GDI, independently of the nucleotide loaded on the protein, and enhances the ability of GDI to extract RhoA from membrane (Ellerbroek et al., 2003; Forget et al., 2002). Subsequently, the other members of the Rho protein family have been shown to be regulated by serine or tyrosine phosphorylation. Cdc42 was phosphorylated at Y64 by Src and this phosphorylation results in the increased interaction between Cdc42 and GDI (Tu et al., 2003). RhoE is phosphorylated at S11 by ROCK 1 and this phosphorylation cause the cytosolic relocation and increased stability of RhoE (Riento et al., 2003).

It was also shown that Rac1 is phosphorylated at S71 by Akt (Kwon et al., 2000). The phosphorylation of Rac1 inhibited its GTP binding activity without any significant change in GTPase activity. Both the GTP-binding and GTPase activities of mutant Rac1 S71A were abolished regardless of the treatment of Akt (Kwon et al., 2000). Therefore, it would be interesting to investigate the roles of phosphorylations on Rac1. Since Rac1 GTP-binding and GTPase activities of Rac1 is not altered by phosphorylation of S71, it would be possible to test other regulatory properties of Rac1, such as whether the binding affinity for GEF, GAP or GDI is affected, or whether the localization of Rac1 is affected.

In this study we successfully demonstrated PLC-γ1 is a GEF for Rac1 and also established the proline rich motif ^{106}PNTP^{109} as an important regulatory element of Rac1. As discovered previously, Erk phosphorylates the serine or threonine in the dipeptide motif S/T-P. There is some preference for proline at -2 or -3 relative to the phospho-acceptor (Songyang et al., 1996). Thus, Rac1 T108 within the ^{106}PNTP^{109} motif is a likely an Erk phosphorylation site (Fig. 1.6). More interestingly, Rac1 also contain an Erk docking site (D-site) at its C-terminal (Fig. 1.6): ^{183}KKRKRKCLLL^{192}. The core consensus motif of Erk D-sites is $(K/R)_{1-3}$-X_{1-6}-φ-X-φ (where φ is a hydrophobic residue). Together, it is very likely that in response to EGF, Erk phosphorylate T108. Therefore, it would be interesting to identify one more possible phosphorylation site (T108) on Rac1 and together identify the possible kinase Erk.

Chapter 5

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